Temperature Dependence of 4-Hydroxy-2-Trans-Nonenal (HNE), a Toxic Aldehyde, in Coconut, Palm, Safflower and Grape Seed Oils

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ABSTRACT

The temperature dependence of the formation of 4-hydroxy-2-trans-nonenal (HNE), the most toxic aldehyde among the four α, β-unsaturated-4- hydroxyaldehydes, was investigated in commercial coconut, palm, safflower and grape seed oils. These oils were heat treated at different temperatures (165, 185 and 218°C) for different length of heating time, such as 0, 1, 3 and 5 hours. The detection and identification of HNE was by using high-performance liquid chromatography (HPLC) of HNE-DNPH and measured by 378 nm UV absorption.

The four commercial vegetable oils for this study were selected based on their different degrees of unsaturation according to their fatty acid distributions. Experiments such as peroxide value, fatty acid distribution, and trolox equivalent antioxidant capacity (TEAC) assay were performed in the unheated commercial oils to obtain general information on their properties. The thiobarbituric acid (TBARS) assay, measuring secondary oxidation products such as aldehydes, and related carbonyl compounds of oils was conducted at 165, 185 and 218°C heating temperature for 0-6 hours. The oils were heated at 165, 185 and 218°C for 1, 3, and 5 hours to measure the formation of the HNE as 2,4-dinitrophenylhydrazone derivatives using high-performance liquid chromatography (HPLC).

It was found that HNE formation was dependent on the heating temperature, the heating time and the level of linoleic acid concentration in four oils. HNE concentration was found higher heating at 185°C compared to heating at 165°C. At

218°C heating, some decomposition of HNE happened in most of the oils. The highest HNE formation was found in grape seed oil, followed by safflower oil, palm oil and coconut oil.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

The interest in lipid oxidation has grown over years due to its influence on food deterioration and relation to human health. Lipid peroxidation process is very complicated, and the oxidation products are primary and secondary oxidation products. The secondary oxidation products include alkanals, alkenals, alkadienals, α, β- unsaturated-4-hydroxyaldehydes and some other degradation products of fatty acids. Among α, β-unsaturated hydroxyl aldehydes, 4-hydroxy-2-trans-nonenal (HNE) is of particular interest, because of its high reactivity and cytotoxicity. The three main functional groups: C=C double bond, carbonyl group and the hydroxyl group make this aldehyde highly reactive. HNE toxicity is resulting in the interference with proteins, DNA and mechanisms of other biomolecules. Numerous literatures reported the toxicity of HNE, indicating that high level of HNE intake can induce deleterious impact on human health such as atherosclerosis, stroke, and liver disease, Parkinson's disease, Alzheimer's disease and Huntington's disease and other diseases.

HNE is formed during peroxidation of ω-6 polyunsaturated fatty acids (arachidonic and linoleic acid) (9). Therefore, the fatty acid composition of various oils is a key factor which influences HNE formation. Coconut oil, palm oil, safflower oil and grape seed oil are relatively new types of culinary vegetable oils used as frying. And linoleic acid concentration of these four oils is considerably different. The present study has investigated the influence of temperature and the length of heating time on the four vegetable oils with varied unsaturation on the formation of HNE.

The present laboratory has already demonstrated that HNE can be incorporated into food from the frying oil during the frying process (20). Moreover, comparing two different frying temperatures such as 190°C and 218°C on soybean, corn and butter oils, their results showed that higher temperature and longer heating time can accelerate lipid oxidation and the formation of HNE concentration as well (24).

The present project investigated lipid peroxidation changes and HNE formation in four different vegetable oils (coconut oil, palm oil, safflower oil and grape seed oil) heating at 165°C, 185°C and 218°C for 0,1,3 and 5 hours.

2 LITERATURE REVIEW

The literature review contains four parts. The first section introduces lipid peroxidation mechanisms, including primary and secondary lipid peroxidation processes. The second section discusses the formation of HNE and its health concerns. The third section reviews studies on lipid peroxidation measurement methods and the effects of temperature and heating time on the oxidation in fats and oils. The fourth section focuses on recent literatures on coconut oil, palm oil, safflower oil and grape seed oil, including information on their oxidation properties.

2.1 LIPID OXIDATION MECHANISM

Fats and oils are important ingredients in human diets contributing nutrition and flavor. Deep fat frying is a popular process for food preparations and the oil used can enhance the texture and flavor of the fried food. However, lipid peroxidation easily

occurs at relatively high temperatures in the presence of air, which produces a multiplicity of compounds influencing the final quality of the product (1). Oxidation of polyunsaturated fatty acids, can not only produce offensive odors and flavors, it can also influence the quality and safety of foods by forming secondary lipid peroxidation products during processing and cooking (2). It's worth noting that certain secondary oxidized products are potentially toxic and related to the formation of several diseases. Therefore, many researchers have studied not only the products in concern, but also conditions that influence their production. Their studies also focus on the mechanisms of lipid peroxidation procedures.

Lipid peroxidation involves three steps: initiation, propagation and termination (2,3). Main lipid oxidation reactions showed on Figure 1(4).

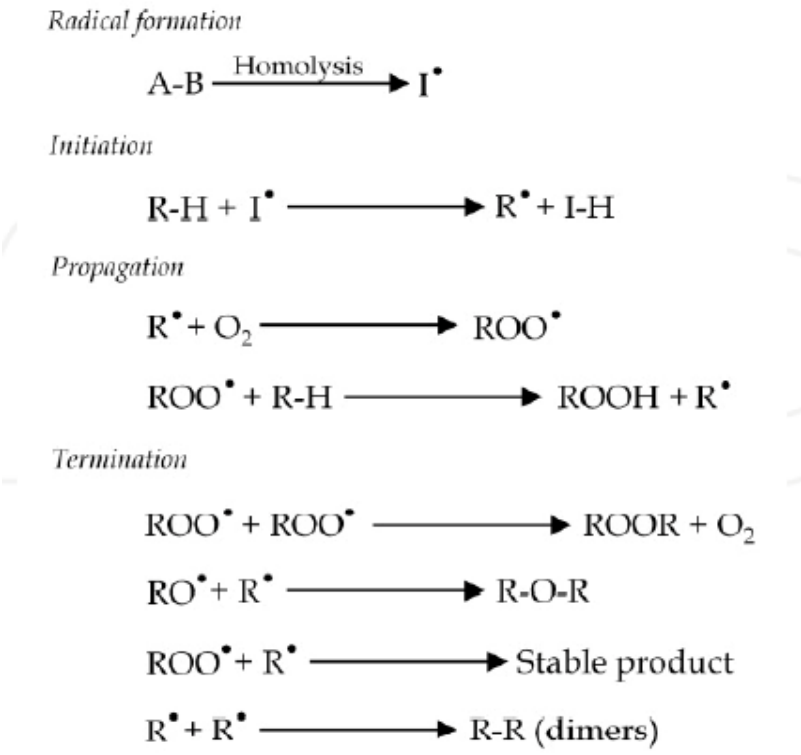

Figure 1: Main lipid oxidation reactions (4)

In the initiation stage, free radicals are formed through several factors, such as temperature, enzymes, UV lights, radiation and singlet oxygen species (4-6). Usually hydrogen loss happened from single methylene interrupted polyunsaturated fatty acid. Oxidation is propagated after abstraction of hydrogen atoms at the α position next to fatty acid double bonds by free radicals, resulting in fatty acid free radical species (7). As the presence of oxygen, oxygen molecule will bind to those attacked fatty acids immediately and produce peroxy free radicals. Then, peroxy radicals in fatty acids will grab hydrogen atom from another fatty acid or methylenic groups of other molecules, which will cause more new free radicals formation (6,7). In this propagation process, primary oxidation products, hydroperoxides formed. And new

unstable free radical groups react with oxygen, and the sequence of the reaction is repeated as above.

Figure 2 shows a generalized scheme for autoxidation of lipids (7).

A variety of volatile and nonvolatile secondary lipid oxidation products are produced after decomposition of hydroperoxides (2, 6, 7). Decomposition of hydroperoxides is a very complicated process and produces multiple compounds that may have biological effects influencing the quality of foods (3). Hydroperoxides decompose as soon as they are formed. The decomposition undergoes scission of oxygen-oxygen bond of hydroperoxides yielding alkoxy radical and hydroxyl free radical very quickly that further reacts to form stable dimer-like products (4, 6, 7).

Homolytic cleavage makes a priority during decomposition of hydroperoxides. As a result, the propagation stage can move on and on and produce more free radicals. And because hydroxyl free radical is extremely active, it will keep grabbing hydrogen atoms to achieve stable state. On the other hand, alkoxy radical can either grab hydrogen atom or the entire fatty acids will decompose. In general, cleavage on the acid side results in formation of an aldehyde of an acid, while scission on the hydrocarbon side yields a hydrocarbon and an oxoacid (6-7). Decomposition will continue happen, letting more chain breaking down, and secondary lipid oxidation products: aldehydes, ketones, hydrocarbons and alcohols and a variety of other related compounds are generated. These stable polymers represent the termination stage of oxidation (4). However, these secondary lipid oxidation products are

responsible for rancid and off-flavors, causing loss of quality and nutritional value of foods. Furthermore, hydroperoxides and some of their breakdown compounds can interact with proteins, enzymes and bio-membranes (3). These reactions with biological components are of great concern since they are influencing vital functional cells, which relate to several diseases.

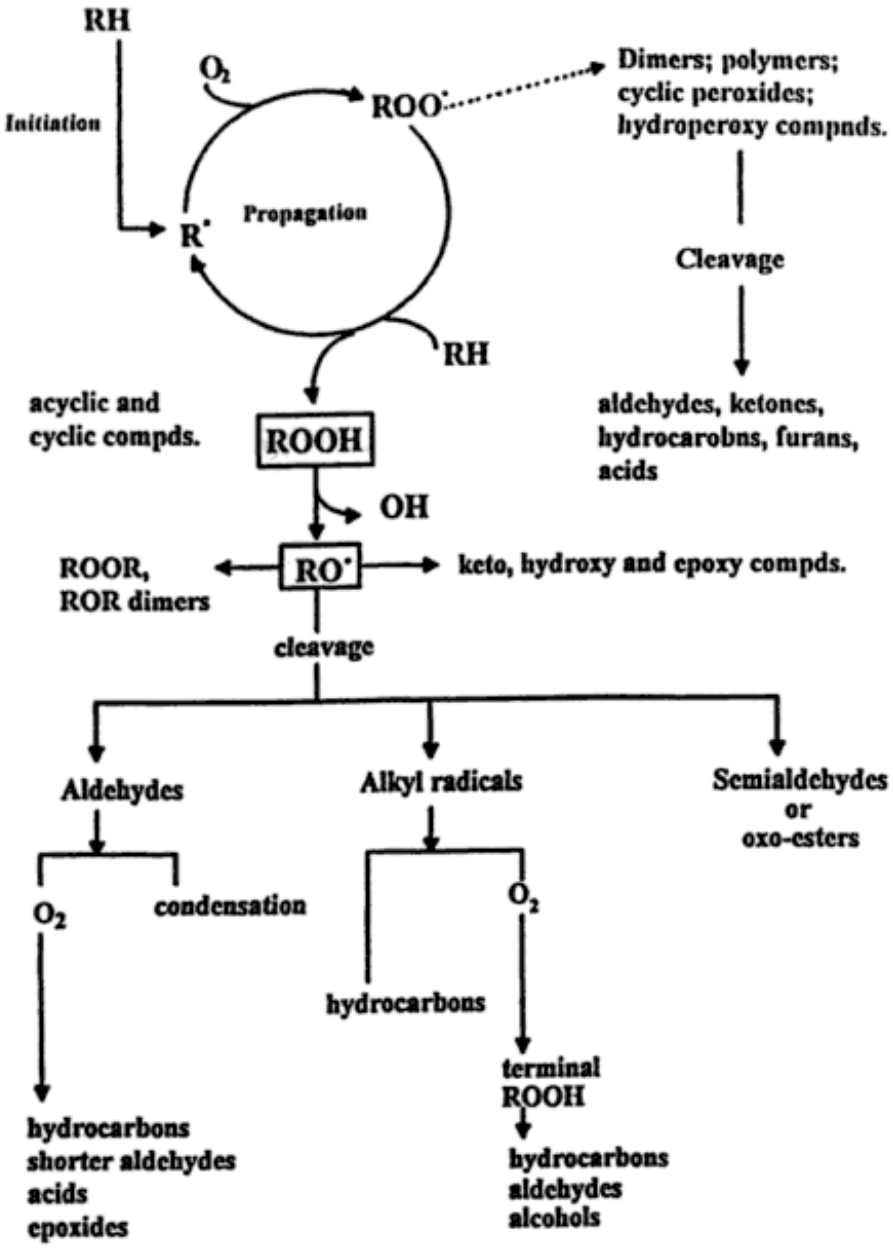

Figure 2: Generalized scheme for autoxidation of lipids (7)

2.2 HNE, 4-HYDROXY-2-NONENAL FORMATION MECHANISM AND HEALTH CONCERNS

HNE, 4-hydroxy-2-nonenal, is one of the well-known toxic secondary lipid oxidation products. In the early 90s, Esterbauer and his colleagues had already brought forward

that hydroxyalkenals, which generated from omega-6 fatty acids, were found cytotoxic and were able to induce cell injury. In addition, Esterbauer et al. also identified that HNE was the major toxic compound among hydroxyalkenals (8). Due to its high reactivity and high toxicity, HNE was analyzed intensively by a variety of investigators.

HNE can be produced by enzymatic reaction or nonenzymatic processes (9). HNE can be formed from enzymatic transformation of omega-6 polyunsaturated fatty acids, such as arachidonic acid, linoleic acid and others, by 15-lipoxygeneases (15-LOX). And in nonenzymatic process, several pathways have been found to explain the formation of HNE. Spickett C recently reviewed the mechanisms of formation of HNE (10). It described that earliest suggestion on HNE formation was based on the decomposition of hydroperoxides to a lipid alkoxy radical by metal ions, such as Fe^{+}, followed by β-scission. For example, 9/13-hydroperoxyoctadecadienoate(HPONE) from methyl-linoleate or 11/15-hydroperoxyeicosatetraenoate (HPETE) from methyl-arachidonate. Later on, Pryor and Porter found out Hock cleavage, which the protonation of the acidified hydroperoxides undertook a rearrangement of C-C to C-O bond, and hydrolysis occurred (9-11). An alternative mechanism was proposed by Schneider et al, who used 9- and 13-hydroperoxides of linoleic acid as starting material, and found two pathways that can support that 4-hydroperoxy-2E-nonenal (4-HPNE) was the intermediate precursor of HNE (9-13). They provided evidence that allylic hydrogen abstraction at C-8 of 13S-HPODE (hydroperoxyoctadecenoic

acid), which leads to a 10, 13-dihydroperoxide that undergoes direct hock cleavage between C-9 and C-10, yielding 4S-HPNE. Moreover, Schneider and his colleagues described another pathway to form HNE, whereas 9S-HPODE cleaves directly following a Hock rearrangement to produce 3Z-nonenal as a precursor of racemic 4-HPNE (12). Figure 3 described two pathways to form 4-HPNE, an immediate precursor of HNE (13).

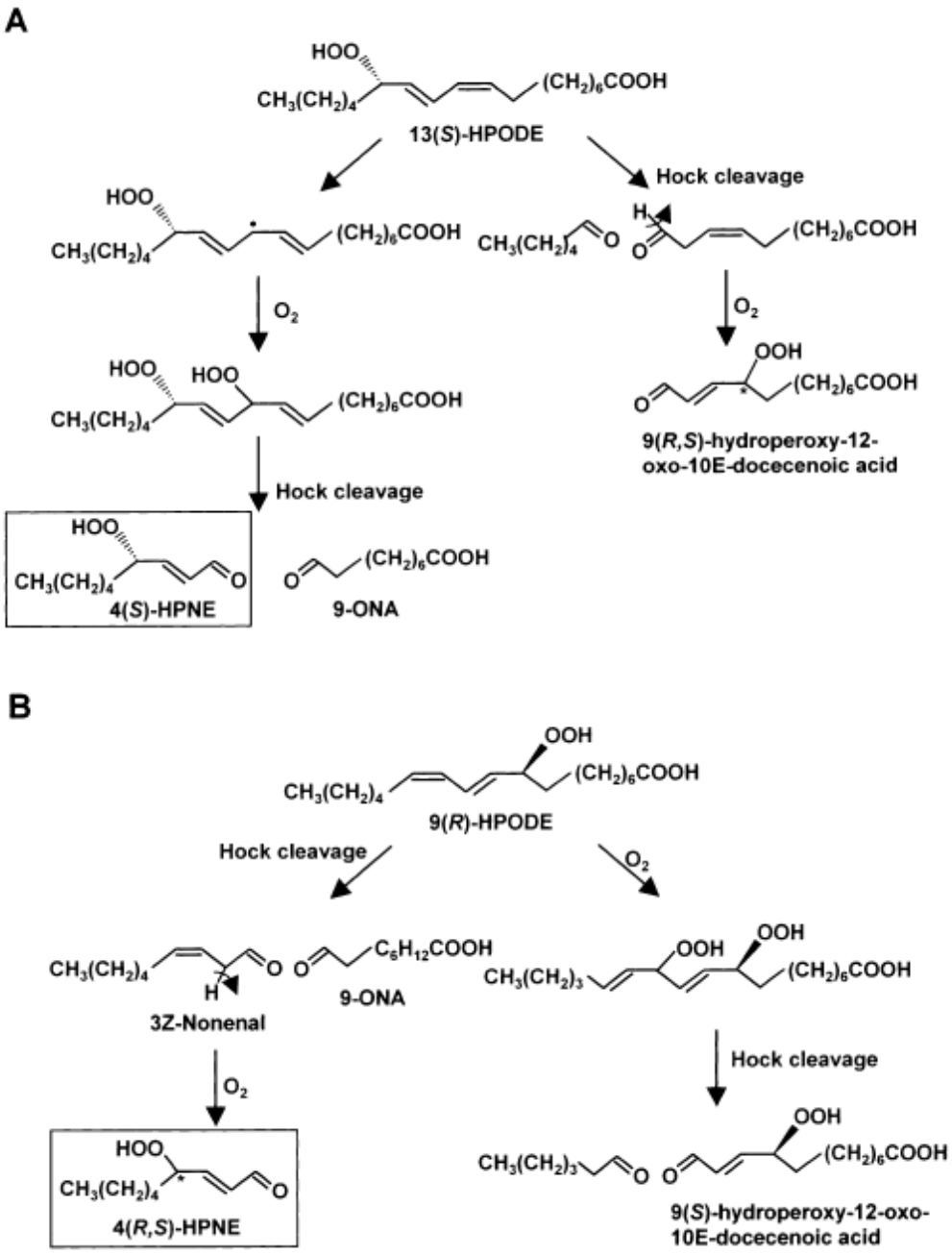

Figure 3: Mechanism of formation of HNE from 13(S)-HPODE (A) and 9(R)-HPODE (B) (13)

HNE has three main functional groups: C=C double bond, carbonyl group and the hydroxyl group. C=C double bond can undergo Michael additions of thiol and amino groups, reduction or epoxidation. Moreover, carbonyl group can target to Schiff base formation, oxidation, and reduction. Hydroxyl group can be oxidized to ketones (9).

These highly reactive functional groups are responsible for generating a lot of serious biological damage and diseases. HNE is able to attach to proteins by Michael addition to Cys, His, and Lys residues, causing loss of function and activity of protein (14, 15). In addition, HNE interacts with key amino acids to stimulate or inhibit enzyme functions. It can also act as a signaling molecule to modify the regulation of gene expression, by modulating nuclear factor kappa B and Ap-1 transcription factors, related to oxidative stress response (15). Moreover, HNE is an amphiphilic compound, but with its much stronger lipophilic, making HNE associate with membranes, and it can also diffuse to different cellular compartments and react with different substrates (14). Therefore, HNE was described that it is a potent electrophile reacting with a variety of nucleophilic compounds and it also acts as a stress signaling molecule (14). HNE can accumulate at concentrations of 10μM to 5mM, in response to oxidative stress and influences biological activities (14). Studies have shown that concentration of 1mM of HNE was found to cause significant changes on protein conformation in synaptosomal membranes (14). In general, HNE-induced modification can lead to alteration of proteins, lipid membranes, and nucleic acids, generating several diseases (13-15).

Recently, excellent reviews come out to describe mechanisms of HNE and its relationship to several diseases. Dalleau and his coworkers summarized different aspects of HNE-induced cell death (15). In Parkinson's disease, HNE can form adducts with proteins that were in proteasome system, causing its failure and neuronal

cell death. Moreover, HNE has been found in mitochondria and Lewy bodies, and it can contribute to mitochondrial dysfunction and degeneration of dopaminergic cells. On the other hand, accumulated HNE has been shown to cause atherosclerotic lesions in both humans and animals. And in Alzheimer's disease, HNE-adducts to neurofilaments has been found, which can prove that HNE performed oxidative damage on neuronal cell bodies (15). What's more, HNE can bind to guanine bases, leading to cancer by inhibiting DNA repair or by promoting inflammation (15). HNE can also contribute to apoptosis, which was a major way of cell death. It is not only a mediator of apoptosis; it can also directly induce apoptosis through extrinsic and intrinsic pathways (15).

Jaganjac and his colleagues summarized on HNE and its function as second messenger of free radicals in diabetes mellitus. The review focused on its reaction as both signaling molecules and as cytotoxic products, and it also described that HNE and oxidative stress worked on beta-cell dysfunction and death during the formation of diabetes mellitus (16). Furthermore, Chapple et al summarized mechanisms of HNE induction on vascular endothelia and smooth muscle cells, which can lead to vascular diseases, such as atherosclerosis, diabetes, neurodegenerative disorders and pre-eclampsia. It also reported that 1-20 μM levels of HNE was found in disease states, while free HNE concentrations in the plasma of healthy individuals range was between 0.3 and 0.7 μM (17).

Zhong and Yin reviewed on the important role of HNE and pathogenesis of cancer and involvement of mitochondria. It showed that HNE can be generated from oxidation of mitochondria-specific phospholipid cardiolipin as well and it also described HNE's relationship to covalent modification of mitochondrial proteins, lipids, and DNA (18). Besides, an important *in vivo* study was conducted by Keller and his colleagues. They used radioactive and stable isotope labeling to analyze how HNE was metabolized after oral administration. Results showed that 48% of the administered radioactivity was excreted into urine and 15% into feces after 24h, and 3% were observed in intestinal and 2% were mostly found in the liver. And for identification of HNE, they found the major peak was identified as 9-hydroxy-nonenoic acids, followed by HNE mercapturic acid derivatives and conjugation (19). This *in vivo* study gave us a good understanding on specific HNE urinary metabolites and its oxidation pathways.

In general, large quantities of researches have shown the high reactivity and cytotoxicity of HNE. New discoveries about the involvement of HNE mechanisms and related diseases and the way to control lipid peroxidation are progressing rapidly.

And in our lab, Seppanen & Csallany (20) found that a considerable concentration of HNE formed at 2h when heating soybean oil at 185°C. And the concentration of HNE increased during 4h and 6h heating, while no HNE detection in unheated soybean oil. The result indicated that heating time is one of the major factors that can influence HNE formation. Moreover, the same author also found that HNE can be incorporated

into food fried in thermally oxidized soybean oil. It brought up a serious concern that high amount consumption of these fried foods can lead to HNE consumption with foods, which causes several diseases (21). Moreover, in our lab, Dr. Csallany and her coworkers also figured out that there was no significant difference after heating soybean oil intermittently or continuously. The formation of HNE and other hydroxyaldehydes at frying temperature was cumulative results of oxidation and the increasing rate between two heating ways was similar (22). Besides, they compared HNE concentration in French Fries purchased from six local fast food restaurants, and the results showed clearly that HNE was produced during heating process of frying oils and was incorporated into French Fries (23). HNE formation was also dependent on temperature. Based on Han & Csallany (24), authors investigated HNE formation in corn, soybean and butter oils heating at 190°C and 218°C. Results showed that HNE concentration at higher temperature was about 5 times higher than at lower temperature, indicating that HNE formation was dependent on temperature.

Therefore, temperature and composition of oils are key factors that can influence lipid oxidation, which shows a great value for scientists to work on it. Saturated fatty acids have been shown to cause several cardiovascular diseases, however, more and more researches found out that high level of polyunsaturated fatty acids are more potent for lipid oxidation, which can result in production of aldehydes, yielding a lot of oxidative stress and diseases.

2.3 Recent Studies on Frying Temperature, Heating Time and Lipid Oxidation

Deep frying is a process of food submerged in hot oil with a contact among oil, air, and food at a high temperature of 150-190℃ (25). Frying time and frying temperature are two key factors that influence thermal oxidation. In general, high frying temperature and longer frying time accelerate thermal oxidation (25).

Lipid oxidation can be measured with physical methods such as color, refractive index, viscosity, specific gravity and dielectric constant. All the data can represent the polymerization of oils and dielectric constant shows that oxygen incorporated into oil (1). Spectrophotometry is used to detect certain chemical substances, such as conjugated dienoic and trienoic fatty acids (1). In addition, chemical methods include iodine value, saponification value, p-anisidine value, peroxide value, and thiobarbituric acid (TBA) test are used to determine lipid oxidation (1). Among them, peroxide value is less useful in the case of heated oils because peroxides decompose spontaneously above 150℃ (26). And TBA test is widely used to detect secondary lipid oxidation. Besides, high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) are commonly used to identify specific oxidized compounds.

Marinova and his coworkers detected oxidative degradation of different thermal heated vegetable oils by monitoring their peroxide value, oxidation stability, conjugated dienes and trienes concentration, changes in fatty acid composition and

content of total polar components (26). In general, total polar components increased throughout the heating period, and triene content increased during the heating time. Moreover, oxidation stability decreased in all oils with increasing heat treatment. All the results shown above indicates that lipid oxidation accelerates throughout heat treatment. On the other hand, Lise and Bente (27) demonstrated the similar results as well. They investigated peroxide value and alkenal concentration on twelve heated (225℃) vegetable oils, results showed that after heating vegetable oils, a large increase in alkenal concentration in all oils. Peroxide values varied in oils since it only shows primary lipid oxidation products, and hydroperoxides in some oils may already decompose to secondary oxidation products (27). Poiana et al studied on thermo-oxidative stability of soybean oil during convective heating at simulated frying temperature. And results have proved again the relationship between heating and lipid oxidation. In the paper, it showed that soybean oil exposed at high temperature accelerates p-anisidine value, total oxidation value. And the content of conjugated dienes and conjugated trienes significantly increased in response to heating (28).

Prabhu (29) determined the level of aldehydic products in different culinary oils, with or without thermal treatment, commercial frying or domestic frying by using thiobarbituric acid method. And he found out thiobarbituric acid activity was much higher in oils rich in polyunsaturated fatty acids than those contained high level of saturated fatty acids or monounsaturated fatty acids, no matter with or without

thermal stress. Moreover, as expected, results showed that lipid peroxide levels were influenced by thermal stress. Furthermore, higher lipid peroxide levels under commercial frying in the oils collected from hotels and caterers were observed compared to the peroxide levels in the oils cooked by domestic frying. The higher results in commercial oils from outside caterers could be due to the habit of using repeated same oils.

Numerous studies have indicated the close relationship between heating and lipid oxidation. Frying temperature and heating time are shown to influence lipid oxidation in various methods.

Tyagi and Vasishtha (30) investigated physical and chemical characteristics and composition of two kinds of oils, and they found numerous changes on both physical and chemical characteristics due to high temperature deep-fat frying. Refractive index, specific gravity, color, viscosity, saponification value of soybean oil increased with rising frying temperature, whereas the iodine value decreased. Since iodine value indicated the unsaturation level of oils, higher frying temperature can increase the destruction of double bonds by oxidation, scission and polymerization, resulting in loss of unsaturation. The same trend was also found in Vanaspati, but less markedly results than soybean oil. The reason is because soybean oil contains high content of polyunsaturated fatty acid, which is easier oxidized with higher temperature. In addition, the results also showed that higher quantities of conjugated dienes were yielded due to higher temperature of frying, and conjugated fatty acids increased from

initial level. Trans fatty acids, which were absent in initial oil samples, were observed in soybean oil samples after 70h of frying at 170, 180 and 190°C.

Another study was conducted by Coscione and Artz (31), they investigated thermoxidative stability of partially hydrogenated soybean oil, by heating the samples at 120, 160, 180 and 200°C continuously for 72h and sampled every 12h. Results presented that acid value, p-anisidine value, color, dielectric constant and the triacylglycerol polymer content of oil samples were dependent on heating temperature and heating time. And the value of each oxidative index increased with increasing temperature and heating time.

A similar result was shown in Chung and Eiserich et al (32). Chung and his colleagues used gas chromatography and gas chromatography/mass spectrometry to identify 99 volatile compounds in headspace samples of peanut oil heated to 50, 100, 150, and 200 °C for 5 hours. Results indicated that total amount of all identified volatiles increased as the temperature of oil was increased. In addition, in Greece, Houhoula and Oreopoulou et al (33) tested thermoxidative alterations of cottonseed oil during frying of potato chips, in the temperature range of 155-195°C. The authors found out that the content of polar compounds, conjugated dienes, conjugated trienes, and p-anisidine value increased with increased temperature.

Moreover, Aladedunye and Przybylski (34) investigated on degradation and nutritional quality changes of canola oil during frying. The authors heated French fries in canola oil intermittently for 7h over 7 consecutive days. The heating

temperature was 185±5 and 215±5°C. They measured total polar components, anisidine value, color components formation, and changes in fatty acid composition and tocopherols to study the changes in canola oil. And they found out that total polar components, anisidine value, color and trans fatty acids content increased with increased frying temperature and time. Furthermore, they also found the polyunsaturated fatty acids decreased with higher temperature and longer heating time, which was due to degradation of polyunsaturated fatty acids. The results also showed that tocopherols degradation increased as a function of frying temperature.

Extensive studies have been worked on temperature and heating time influence on lipid oxidation. However, there are very limited studies on some new commercial vegetable oils that are commonly used nowadays. My research is to investigate lipid oxidation changes on four different vegetable oils: coconut oil, palm oil, safflower oil and grape seed oil by heating oils at 165°C, 185°C and 218°C.

2.4 RECENT RESEARCHES ON COCONUT OIL, PALM OIL, SAFFLOWER OIL AND GRAPE SEED OIL

2.4.1 Coconut oil

Coconut oil is commonly used in South Asia, and it contains a high level of low molecular weight saturated fatty acid, the major fatty acid is lauric acid (35). There was a long time that scientists believe that coconut oil can elevate blood cholesterol since it contains mostly saturated fatty acids. However, recent studies have shown the beneficial effects of coconut oil on lipid oxidation. Most commercial coconut oils are

produced by crushing copra, and use sun drying or smoke drying to get final product. In contrast, virgin coconut oil is extracted from wet process directly from coconut milk under controlled temperature (35). Coconut oil contains about 92% saturated fatty acids, making it very stable and having a long shelf life (36). Maria et al investigated chemical properties on virgin coconut oil and found that the low iodine value indicated that virgin coconut oil had high degree of saturation. Moreover, they also studied on peroxide value and anisidine value; both results were relatively low, showing that coconut oil is very stable against oxidation (35).

Moigradean and his colleagues also investigated on quality characteristics and oxidative stability on coconut oil during 12 months of storage (36). They analyzed peroxide value, anisidine value, and total oxidation value. Results presented that the peroxide value was very low during 12 months of storage, which implied that coconut oil had high oxidative stability. And peroxide value increased from month to month, but after 9 month the values decreased because secondary oxidation products appeared. Moreover, anisidine value were in the range 0.19-0.87, which supported again that coconut oil had high stability on lipid oxidation due to its high level of saturated fatty acid, and it was easily to control good quality for a long shelf life. Claxson et al presented similar results monitored by high field H NMR spectroscopy. They heated oils for 30-90mins at 180 C and results indicated commercial coconut oil generated very low levels of specific aldehyde (37).

On the other hand, animal studies were used to analyze the relationship between coconut oil and lipid peroxidation. A recent study was conducted to figure out the beneficial effects of virgin coconut oil on lipid oxidation parameters and in vitro LDL oxidation (38). The authors fed oil to Sprague–Dawley rats for 45 days in order to determine several lipid oxidation parameters and lipoprotein levels. Results showed that virgin coconut oil was able to decrease total cholesterol, triglycerides, phospholipids, LDL, and VLDL cholesterol levels and increase HDL cholesterol in serum and tissues, which indicating that there are a lot of beneficial effects of consuming virgin coconut oil to lower lipid oxidation levels in serum and tissues and LDL oxidation by physiological oxidant. Another study was conducted on supplementation of the diet of female F344 rats with several oils, investigating the formation of the promutagenic, exocyclic DNA adducts in the liver (39). Results indicated that coconut oil did not increase body weight compared to high MUFA and PUFA diets. Besides, rats supplemented with coconut oil resulted in the lowest HNE-dGp, which are the predominant DNA lesions caused by lipid peroxidation product HNE. Likewise, an animal study focused on investigating the oxidative response of rat liver microsomes by feeding growing male rats on 15% diets containing either soybean oil, olive oil or coconut oil (40). After 6 weeks, microsomes from coconut-fed rats had the highest content of saturated fatty acids, and the aldehyde rate production was low in coconut-fed rats. HNE was undetectable in microsomes of coconut-fed rats, which means coconut-fed rats were the most resistant to lipid peroxidation.

Antioxidant activity of coconut oil has been an interest in some researches too. Janu et al investigated the antioxidant potential of commonly used vegetable oils, including coconut oil. Results showed that the percentage of total phenolic content (TPC) of coconut oil was 1.8mg GAE (gallic acid equivalent)/100g oil, which had the second highest percentage TPC. And by analyzing DPPH radical scavenging activity, coconut oil showed the highest activity (41). Nevertheless, another research illustrated coconut oil had very low content of natural antioxidants, and therefore a very low radical scavenging activity (42).

2.4.2 Palm Oil

In recent times there has been a growing research interest in palm oil. Palm oil is usually used in tropical area and palm oil contains almost equal proportions of saturated and unsaturated fatty acids. The unique composition of palm oil is that it contained high amount of palmitic acid as well as large proportions of oleic and linoleic acid, giving it a much higher unsaturated fatty acid content than coconut oil (43).

Edem reviewed on the physiological, hematological, and toxicological aspects of palm oil consumptions (43). He analyzed large quantities of animal experiments on investigating palm oil consumption and cholesterol level and he found that consumption of palm oil in normal diets reduced plasma cholesterol level. Moreover, palm oil was found to have a protective effect on the endothelium of the blood vessels. And platelet aggregation, arterial thrombosis tendency have been found to be

decreased by palm oil diets (43). Palm oil showed protective effect against oxidative damage of the liver and tumorigenesis as well (43). In addition, Ebong and his colleagues also published a review paper on palm oils and they summarized that fresh palm oil was beneficial to the body in terms of low heat production and it does not adversely affect body weight and morphology of various tissues (44). They also presented that fresh palm oil was able to help reduce the blood levels of cholesterol, triglycerides, LDL-cholesterol and other lipids distributed in the body (44). Another review paper indicated that palm oil was as healthful as olive oil based on its high monounsaturation at the crucial 2-position of the oil's triacylglycerols (45). Palm oil was considered to have high stability to free radical oxidation due to its high content of monounsaturated fatty acids and its natural antioxidants content such as tocotrienols (45). A number of human feeding studies were conducted in pre-1990 and their results showed that palm oil diets reduced blood cholesterol values ranging from 7% to 38% (45).

Bracco et al (46) compared frying performance of palm oil compared with other vegetable oils. Results indicated that palm oil was satisfactory as a frying medium by comparing different analytical parameters such as smoke and flame point etc, and it showed a lower increase of polymers, viscosity and foam formation. Moreover, Man and Hussin (47) investigated frying performance of palm oil comparing with coconut oil. Results showed that palm oil was superior in terms of its parameters like % free fatty acid, iodine value, foaming tendency and smoking point. Czerniak et al (48)

evaluated antioxidant capacity of palm oil by two different refining processes. By using FRAP and DPPH method, the antioxidant capacity of palm oil can be described as 19.5–102.8 μmol TE (Trolox equivalent) /100 g and 18.8–103.0 μmol TE/100 g from mode one process. And the antioxidant capacity of palm oils from mode two process was higher than the AC for palm oils from mode one process.

In early 2000s, numerous studies have confirmed the nutritional value of palm oils, however, the studies were all analyzed on fresh palm oil. However, there were growing researches showed that the used and thermally oxidized palm oil had deleterious effects on human health.

A vivo study was conducted by Adam and his coworkers to investigate on the effects of repeatedly heated palm oil on serum lipid Profile, lipid peroxidation by using post-menopausal rat model (49). Twenty-four female rats were divided into four groups. The control group was given 2% cholesterol diet only throughout the study period. The three treatment groups received 2% cholesterol diet fortified with fresh, once-heated or five-times-heated palm oil, respectively. Results showed that five-times-heated palm oil caused a significant increase in TBARS and total cholesterol (TC) compared to control. They also found that a great increase in serum homocysteine in the control and five times heated palm oil group compared to fresh and once-heated palm oil groups. The findings implied that repeatedly heated oils had the major impact on the development of atherosclerosis.

A similar study was conducted by Falade and his colleagues (50). The author assessed the biochemical responses of rats to thermally oxidized palm oil diets. They fed rats with fresh palm oil (control group) and thermally oxidized palm oil (test group) for 30 days. And they found that there was a significant decrease in the plasma and liver total protein, albumin, triglyceride and high-density lipoprotein of the test groups when compared with the control. On the other hand, there was a significant increase in the activities of alanine transaminase, aspartate transaminase and alkaline phosphatase, total bilirubin, total cholesterol and low-density lipoprotein compared to control diet. The results showed obvious difference on fresh palm oil and thermally oxidized palm oil, and indicated that consuming thermally oxidized palm oil can cause several detrimental diseases.

2.4.3 Safflower oil

Safflower oil contains more than 70% polyunsaturated fatty acids and it can oxidize easily by atmospheric environment (51). Among abundant of polyunsaturated fatty acid in safflower oil, linoleic acid is the main fatty acid. The fatty acid composition of safflower oil gave us a general idea on how easily and fast safflower oil will undergo oxidation with its high contents of polyunsaturated fatty acid.

Khatoon and Krishna assessed the oxidation of thermally heated safflower oil by physical, chemical and spectroscopic method (52). They heated safflower oil in an open pan or in an oven, or by deep frying for 8h. Results showed that heating in open pan caused deterioration in oil faster than in other treatments. The viscosity of

safflower oil increased sharply after 15-30 min of heating followed by a considerable increment in viscosity after 3h of heating, which can be due to polymerization. Moreover, heating resulted in reduction in iodine value in all of the treatments of safflower oils. By analyzing infrared (IR) and nuclear magnetic resonance (NMR) spectroscopic techniques, they analyzed the extent of oxidation. They found conjugated dienes rose, and –OH group formation due to peroxide breakdown, generation of aldehydes and ketone, and molecular aggregation due to polymerization. As expected, all the compounds found produced by lipid oxidation mechanisms.

Likewise, Muik et al and Lee et al both investigated safflower oil relation to temperature and oxidative stability (53, 54). Results presented that oxidative degradation of safflower oil was accelerated by heating, and also with higher heating temperature. In addition, Bozan and Temelli studied on chemical composition and oxidative stability of flax, safflower and poppy seed oil (55). The study found that safflower oil contained 70.5% linoleic acid, which was in accordance to other studies. And they also tested total tocopherol content and found 12.1mg total tocopherols were presented in 100g safflower seed. By comparing oxidative stability among three oils, they demonstrated that safflower was in the middle between poppy oil and flax oil.

Animal studies were also conducted to investigate the property of safflower oil; however, most studies were using safflower oil to compare with other oils. Shimomura and his coworkers worked on the effects on fat accumulation in rats fed

either a safflower oil diet or a beef tallow diet for 4 months (56). Results showed that oxygen consumption was significantly greater in the safflower group than in the beef tallow group, indicating greater thermogenesis in the former group. Besides, by assessing respiratory quotient, the fat oxidation rate was higher in the former group as well. Body fat accumulation was less in rats fed in safflower oil, and serum triacylglycerol level was significantly lower in the safflower group compared to the beef tallow diet group. These results suggested that the consumption of safflower oil can lower serum triacylglycerol level, due to its high content of unsaturated fatty acids. Another animal study was focused on the effects of fish and safflower oil on lipoprotein metabolism in perfused rat liver (57). By analyzing several enzyme activies and rates of hepatic lipogenesis and ketogenesis, results indicated safflower oil had less effect on lowering plasma triacylglycerols compared to fish oil diet.

2.4.4 Grape seed Oil

Grape seed oil is produced from the seeds in the pomace waste from juice and wine production, which is valuable for complete utilization of grapes (58, 59). It's necessary and critical to dry the residue of wine or juice very fast to achieve high value grape seed oils with great smell and taste as well as polyphenolic compounds (59, 60). Because during pressing, the pomace is composed of seeds together with fruits containing a lot of moisture, which makes the residue very susceptible to microorganisms. Moreover, enzymes in the pomace start working and both microorganisms and enzymes can develop unpleasant aroma pass over into the oil during pressing (60).

Pomace contains approximately 20-26% grape seeds, 7.8-11% protein and 10-20% fatty oil depending on pressing conditions (59). Grape seed oil is gaining popularity as a culinary oil, which is composed of average 90% polyunsaturated and monounsaturated fatty acids, particularly of linoleic acid (58-78%) followed by oleic acid (3-15%) (58, 59). The content of high level of polyunsaturated fatty acids brought great interest by this study to analyze its lipid peroxidation.

Lutterodt investigated the fatty acid composition, oxidative stability and antioxidant properties of selected cold-pressed grape seed oils (58). The authors demonstrated that the most abundant fatty acid in the oils was linoleic acid, which was in agreement with other studies in literatures. In addition, DPPH radical scavenging capacity ranged from 0.07 to 2.22 mmol trolox equivalents/g of oil. And oxidative stability index was measured in various kinds of grape seed oils, showing that oxidative stability of grape seed oils ranged from 40h to 19.69h, which was less than 46.51h recorded for commercial soybean oil under the same experimental conditions.

Bail et al reported the characterization of various grape seed oils by investigating volatile compounds, triacylglycerol composition, total phenols and antioxidant capacity (59). Results showed that total phenol content ranged between 59μg/g and 115.5μg/g GAE. Antioxidant capacity was analyzed and found to be between 0.09 μg/g and 1.16 μg/g. Besides, they research concluded that virgin grape seed oils produced from grapes of red wine production contain a higher number of volatile compounds, and the total phenol content and trolox equivalent antioxidant capacity

(TEAC) were higher, compared to grape seed oils from grapes of white wine production.

Phenolic compounds in grape seeds have been of more recently focus on their antioxidant capacity (60-62). Gallic acid, catechin and epicatechin and a wide variety of procyanidins have been detected in grape seeds. Base on Maier et al, the total polyphenol of the seeds ranged from 107.4 to 226.0g gallic acid equivalents (GAE)/ kg seed. And the TEAC values of crude extracts from grape seeds ranged from 48.49 to 104.8 mol TAE/110g DM (61). However, a review paper doubted the high amounts of phenolic compounds in the grape seed oil and said that large quantities of these compounds can be found in the seeds, but during oil pressing process, the main amount of phenolic compounds remains in the residue, because their solubility in oil is limited (60). The statement was in accordance with the results shown above, which showed that the antioxidant capacity and phenolic content were much lower in the grape seed oil compared in the crude extract from seeds.

Pilehvar and his collegues studied the effect of grape seeds oil on lipid content of LDL, HDL, cholesterol, TG and VLDL of serum in rats (63). 20 rats were divided into 4 groups, one control group which received a standard diet and other 3 groups were fed with grape seeds oil at a dose of 100mg/kg daily besides of standard diet for 1, 4 and 8 weeks, respectively. Results showed that grape seeds oil was able to decrease triglyceride after 8 weeks treatment but its value in 8 weeks control groups was increased significantly. Besides, cholesterol levels in 8 weeks control group was

significantly higher than other groups. However, VLDL and LDL showed no significant difference during the study. HDL showed significant increase 2 month after treatment with grape seeds oil. The results implied promising influence of grapeseed oil on decreasing the TG and cholesterol and increasing the HDL. However, there are still other concerns on grape seed oil since it contains high level of PUFA, which can accelerate lipid oxidation, producing more secondary lipid oxidation products during heating and causing more diverse diseases.

3 EXPERIMENTS

3.1 MATERIALS AND METHODS

3.1.1 Chemicals and Instruments

Two brands of coconut oil, palm oil, safflower oil, and grape seed oil were analyzed in the present study. Among them, Brand A coconut oil (Crisco), Brand B coconut oil (LouAna) and Brand A safflower oil (LouAna) were purchased from retail stores (Roseville, MN). Brand A palm oil (Fuji Vegetable Inc.) was sent from savannah, GA. Brand B palm oil (Okonatur), Brand B safflower oil (Spectrum), Brand A grape seed oil (GrapeOla) and Brand B grape seed oil (Baja Precious) were obtained from online store since they are rare to get in local retail stores.

2-thiobarbituric acid, thichloroacetic acid, HPLC-grade water, HPLC-grade methanol, -HPLC-grade dichloromethane, hexane, ethanol and boron trifluoride-methanol, solution were purchased from Sigma Chemical Company (St. Louis, MO); trolox

(6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), ABTS, 2,2'-azinobis (30ethylbenzothiazoline-6-6sulfonic acid) diammonium salt were obtained from Sigma Aldrich. Potassium persulfate, sodium thiosulfate, glacial acetic acid, hydrochloric acid, acetone and hexane solution were from Fisher Scientific (Fair Lawn, NJ); potassium iodide was obtained from Mallinckrodt Baker Inc. (Paris, KY). HNE standard was from Cayman Chemical Co. (Ann Arbor, MI). Moreover, No. 1 filter paper and 0.45um syringe filters were purchased from Whatman Ltd. (Kent, England). Plates for thin layer chromatography (TLC) were from EMD Millipore, Inc. (Billerica, MA). 2, 4-Dinitrophenylhydazine was purchased from Eastman Kodak Co. (Rochester, NY).

The gas chromatograph used a 18835B capillary inlet system (5830A Gas Chromatograph, Hewlett-Packard, Saginaw, MI).

The HPLC system consisted of a sample injector (712 WISP, Waters, Milford, MA), a solvent delivery system (9050, Varian, Walnut Greek, CA) and a UV-Vis detector (9010, Varian). The HPLC column was Ultrasphere ODS (5×4.6 mm, 25 cm, Hichrom, Berkshire, UK). Detailed operating parameters are provided later in the methods section (General Outline of the Methods Used).

3.1.2 Thermal Treatments of the Oil Samples

Duplicate 2g ± 0.05g oil samples were placed in open test tubes (16×150mm) in a sand bath and continuously heated at 165, 185 and 218°C for 1, 2, 3, 4, 5, and 6 hours. Target temperatures were reached in 20 minutes.

3.1.3 Peroxide Value

Peroxide value determination of the unheated oil samples were conducted according to the method of the American Oil Chemists' Society (64). 1 gram of unheated oil (in duplicate) was weighed accurately into Erlenmeyer flask, and then 15ml mixed solution containing 6 parts of glacial acetic acid and 4 parts of USP chloroform was added into it. Later, 1ml saturated aqueous potassium iodide (KI) solution was added and the mixture was swirled gently for 1 minute. After leaving mixture solution in a dark environment for 5 minutes, 15ml water was added and mixed as before. The mixture solution was then titrated with 0.002N sodium thiosulfate until yellow color became faint. Several drops of starch solution were added to show a clear point.

3.1.4 Fatty Acid Distribution by Gas Chromatography

Fatty acid distribution of unheated oils and fats was determined by gas chromatography. In duplicate, 2 drops of each unheated oils or fats was added into a 20ml test tube, and then 3ml BF_3-Methanol (14% BF_3 in methanol) was added. The test tubes were capped and shaken vigorously. Then they were placed in boiling water bath for one hour. After cooling, 3ml distilled water and 10ml hexanes were added into mixture and they were shaken for 10 minutes. After mixture was separated into two layers, the top hexane layer was removed and saved. The hexane samples were dried with 1-2 grams sodium sulfate. At last, 1µl dry hexane sample was injected into GC and the amount of injection was determined by the area and figure of chromatogram. The fatty acid distribution was measured by comparison with the retention times of fatty acid standards.

3.1.5 TBARS Assay

2-ThioBarbituric Acid Reactive Substances (TBARS) method is commonly used for investigating overall lipid peroxidation (65). TBARS assay can detect aldehydes and ketones generated by lipid peroxidation. The reaction gives rise to a pink color, which can be absorbed and detected by spectrophotometer at 530-532nm. Malondialdehyde (MDA) can be used as standards and the test results are expressed as MDA equivalents.

Duplicate samples of 2g (±0.05g) coconut, palm, safflower and grape seed oil were placed in open test tubes (16×150mm) and they were inserted into a sand bath and heated continuously at 165°C or 185°C or 218°C for 1, 2, 3, 4, 5, and 6 hours. It needs 20 minutes to reach the target temperature.

Based on Coudray et al (65), the TBA assay is accomplished by mixing TBA with oil sample in an acid medium and boiling mixture in water bath. In our method, TBA reagent was prepared with equal volumes of 15% w/v trichloroethanoic acid (TCA), 0.375% w/v 2-thiobarbituric acid (TBA), and 0.25N hydrochloric acid. Duplicate of 200µL oils was added into tubes with 4ml TBA reagent, and then placed tubes into boiling water bath for 15min. Absorbance of samples was measured at 535nm by spectrophotometer. The standard curve of MDA was done by the same method. It changed the concentration of MDA to react with TBA reagent in order to get a standard curve.

Table 1: The Concentration of MDA for TBARS standard curve

Tube number	MDA(5×10^{-5}M)(μL)	Water(μL)	Concentration of MDA(μg/mL)
1	0	200	0
2	50	150	0.043
3	100	100	0.086
4	150	50	0.129
5	200	0	0.172

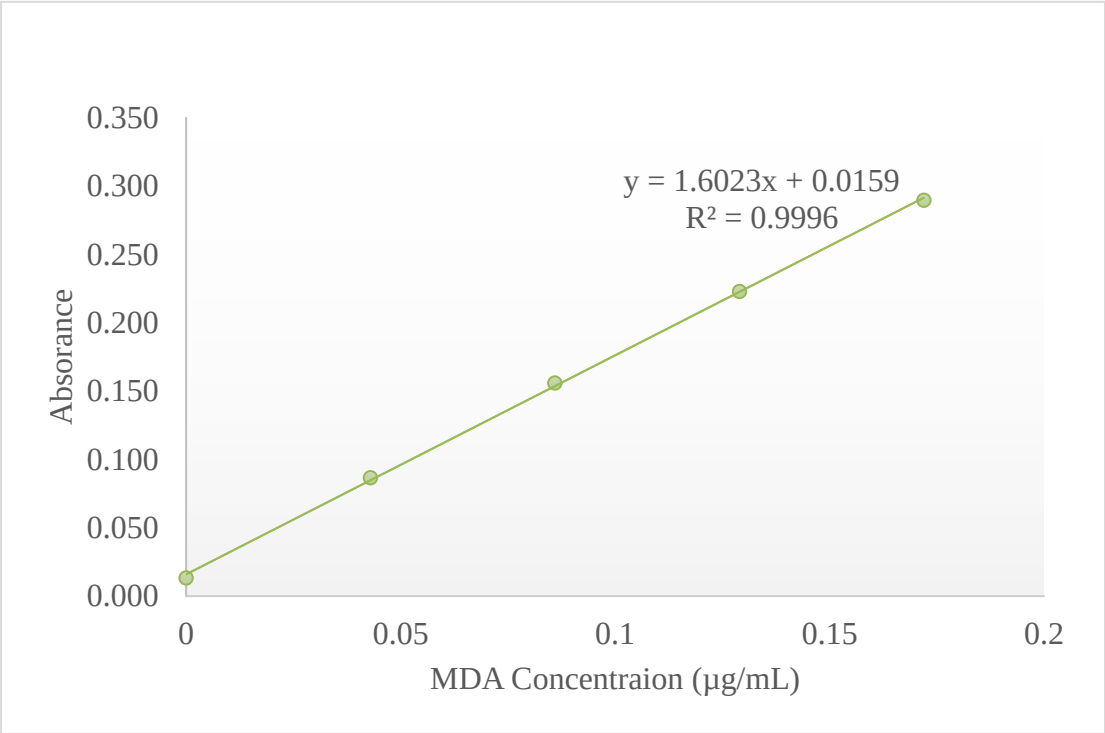

Figure 4： TBARS Determination of MDA Standard Curve (5×10-5M)

3.1.6 Method to Determine the Secondary Oxidation Products of Samples

The principle in this method is using the reactivity of 2, 4-dinitrophenylhydrazine (DNPH). It can react easily with secondary oxidation products such as aldehydes, ketones and related carbonyl compounds. The derivatization of carbonyl with DNPH, can be separated on TLC plates into three groups, polar group, nonpolar group and

osazones. By using reverse phase C18 column HPLC, the analysis of 2,4-dinitrophenylhydrazone of 4-hydroxyalkenals can be detected by ultraviolet light (UV) at 378nm (66).

3.1.6.1 Detailed Methods:

1) **Recrystallization of DNPH**

Two gram DNPH was dissolved in 200ml methanol and heated at 60°C for 1h, and the dissolved DNPH was placed in an ice bath for at least 18h for recrystallization. The crystallized DNPH was filtered by No.1 filter paper and dissolved again in about 200ml methanol. The crystallization process was repeated at least two more times and the collected DNPH crystals were placed in a desiccator for 3 days for drying.

2) **Preparation of DNPH Reagent**

10mg freshly recrystallized DNPH was dissolved in 20ml 1N hydrochloric acid and heated at 50°C for 1 hour. After cooling, the impurity was washed four times with 10ml of hexane and the hexane layers were discarded.

3) **Preparation of DNPH reagent standard and acetone-DNPH standard**

The acetone-DNPH standard was prepared by combining equal volumes of acetone with freshly prepared DNPH reagent, incubating overnight at room temperature. After incubation, the mixture was extracted three times with 5ml dichloromethane. And the solvent was evaporated by N_2 gas until sample size reached 0.5ml. To prepare DNPH reagent standard, added small amount of DNPH into 3ml dichloromethane.

4) Preparation of 2,4-dinitrophenylhydrazones of lipophilic aldehydes and related carbonyl compounds from unheated and heated oils

One gram of heated or unheated oil sample (in duplicated) was reacted overnight at room temperature with 5ml freshly prepared DNPH reagent. The DNPH derivatives were extracted with 10ml methanol/water (75:25, v/v) and then put the combination into centrifuge at 2000rpm for 10min and the DNPH derivatives were extracted with tube. Later on, the DNPH derivatives were re-extracted with 10ml dichloromethane three times from the combined methanol/water extracts and concentrated with N_2 gas to about 1ml.

5) Separation of Polar, Nonpolar Hydrazones and Osazones by Thin-Layer Chromatography (TLC)

The concentrated DNPH derivatives were applied to two TLC places for pre-separation. The sample was applied in a very thin line across the plate with a 250µL micropipette attached to a Hamilton syringe with a piece of flexible rubber tubing. The DNPH reagent standard and acetone-DNPH standard were spotted on the plate for reference. And the plates were placed and developed in dichloromethane.

Polar carbonyl compounds were shown between original and DNPH blank reagent band, and nonpolar carbonyl compounds were between DNPH-acetone band and solvent front. The polar and nonpolar region, were cut from TLC plates into small pieces and extracted from TLC plates three times with 10ml methanol, respectively.

The combined methanol extracts were evaporated under N_2 gas and placed into 1ml volumetric flask to the exact volume of 1ml. The finished samples were sealed in amber vials and stored under -20°C for later HPLC analysis.

6) Separation and identification of DNPH derivatives of polar (PC) and nonpolar (NPC) lipophilic aldehydes and related carbonyl compounds from oils and fats by HPLC

50µl aliquots of polar carbonyl DNPH derivatives and nonpolar carbonyl DNPH derivatives were injected into an HPLC reverse-phase C18 column, equipped with a guard column.

For polar compounds group, 10 min of isocratic elution with methanol/water (50:50, vol/vol) was followed by a linear gradient to 100% methanol for 20 min, then 100% methanol for an additional 10 min at a flow rate of 0.8mL/min. Absorbance was measured at 378 nm.

For nonpolar compounds group, an initial isocratic elution was methanol/water (75:25, vol/vol) for 10 min and was followed by a linear gradient to 100% methanol for 20 min, then 100% methanol for an additional 10 min at a flow rate of 0.8mL/min. Absorbance was also measured at 378 nm.

Pure HNE standard was injected into HPLC every day to check the retention time before analysis of samples. Identification of individual compounds from oils is

accomplished by comparing the retention times of known standards to the retention times of peaks derived from the oil samples.

Co-chromatography was used to help identify and quantify certain compounds. Sample, the standard and the mixture of sample and standard were injected, respectively. The ratio of the peak area of the mixture sample compared with expected peak area was calculated. Therefore, the percent of recovery was obtained. The recovery is calculated and found to be generally between 95% and 110%. Since coconut oil showed close peaks under lower temperature heat treatment and some unheated oils presented uncertain peaks, co-chromatography was used to identify targeted compounds.

The total concentration of the polar and nonpolar aldehydes and related carbonyl compounds were calculated and measured by the peak area of HPLC chromatogram and were expressed as ng hexanal equivalent /g oil. 1 ng hexanal equivalent was 22,182, and the factor was previously determined by using known amount of hexanal-DNPH measuring area by HPLC at 378nm UV absorption.
HNE concentration was expressed as µg HNE/g oil based on the conversion of molecular weight of hexanal (100) to HNE (156). Total PC-DNPH and NPC-DNPH concentration was expressed as µg hexanal equivalent /g extracted fat since the individual aldehyde and carbonyl compounds are unknown.

3.1.7 Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The Trolox equivalent antioxidant capacity (TEAC) assay is an easy and popular method for assessing the antioxidant capacity of a compound to scavenge ABTS radicals (ABTS) (67). It is based on the principle when ABTS is incubated with a chemical, it produces ABTS radical cation (ABTS•+) and the ABTS•+ can provide a blue-green color under UV absorption. Antioxidants in the sample reduce ABTS•+ showing lower UV absorption to a degree that is proportional to their concentrations (68).

For making standard curve:

ABTS was dissolved in water to make 7mM concentration of ABTS. Then, ABTS stock solution was reacted with 2.45mM potassium persulfate to produce ABTS radical cation. Allow the mixture to stand in the dark at room temperature for 12-16h before use. The ABTS•+ solution was diluted with ethanol to an absorbance of 0.7 at 743nm and equilibrated at 30°C. 2.5mM Trolox was prepared in ethanol and used as a stock standard. Different concentrations of Trolox standards were made in ethanol. 4ml diluted ABTS•+ was added into 40µL of Trolox standards, mixed well, triplicated. The absorbance reading was taken after 6min. 40 µL of ethanol in 4ml of diluted ABTS•+ was run as control in each assay. The standard curve was shown as below.

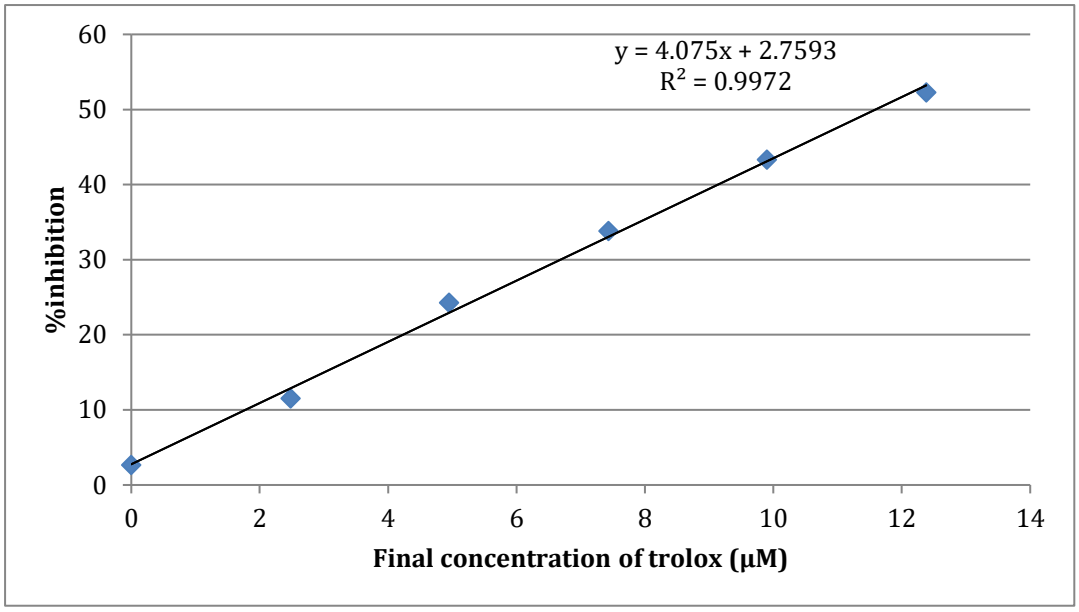

Figure 5: Standard Curve for trolox equivalent antioxidant capacity (TEAC) assay

Calculation:

X axis: Final concentration of Trolox (µM)

Y axis: %inhibition = $(Abs_{control}-Abs_{sample})/ Abs_{control}\times100$

For sample:

1g oil sample was dissolved in 5ml hexane and mixed well. 4ml diluted ABTS•+ solution was added into 40 µL oil sample in hexane, mixed well, triplicated. The absorbance was taken after 6 min. 40 µL of hexane in 4ml of diluted ABTS•+ was run as control in each assay.

3.1.8 Statistical Analysis

All experiments were conducted in duplicates and the data are expressed as mean ± standard deviation. ANOVA was used to determine if there were significant

differences between the groups. A Tukey test was conducted to calculate the p values. Statistically significant differences were determined at $p \leq 0.05$.

4 RESULTS

4.1 PEROXIDE VALUE

Table 2 showed the peroxide value (PV) of 8 unheated commercial vegetable oils with 2 brands in each oil using titrations. The peroxide values were in the range between 1.74-8.90 milliequivalents of peroxide per kg. The range was large since the oils have different fatty acid distribution and the oxidation rate is different as well. In general, fresh oils have a peroxide value of <10 mEq/Kg (69), therefore, all the vegetable oils obtained were shown in fresh status.

Table 2 Peroxide value of unheated vegetable oils

Oil + Brand	Peroxide value (milliequivalents of peroxide per kg)	Oil + Brand	Peroxide value (milliequivalents of peroxide per kg)
Safflower oil A	8.90±0.31	Safflower oil B	5.79±0.24
Grape seed oil A	5.31±0.51	Grape seed oil B	4.69±0.61
Palm oil A	2.07±0.64	Palm oil B	2.50±0.47
Coconut oil A	2.69±0.29	Coconut oil B	1.74±0.50

4.2 FATTY ACID DISTRIBUTION

The fatty acid distributions of commercial safflower oil, grape seed oil, palm oil and coconut oil were measured mainly to determine the saturation of oils and the concentration of linoleic acid in the samples since linoleic acid has been reported as the precursor of the formation of 4-hydroxy-2-nonenal (9-11).

Table 3 shows the retention times of pure lauric, palmitic, stearic, oleic, linoleic and linolenic acid standards used to identify specific fatty acids in oils.

Table 3: Retention times of Fatty Acid by GC Method

Standard	
Fatty acid	Retention time (min)
Lauric acid	1.03
Palmitic acid	1.69
Stearic acid	2.95
Oleic acid	3.19
Linoleic acid	3.75
Linolenic acid	4.66

By comparing the retention times of fatty acids between samples and the pure standards, the fatty acid distributions of two brands of safflower, grape seed, palm and coconut oil in this experiment are shown in Table 3.

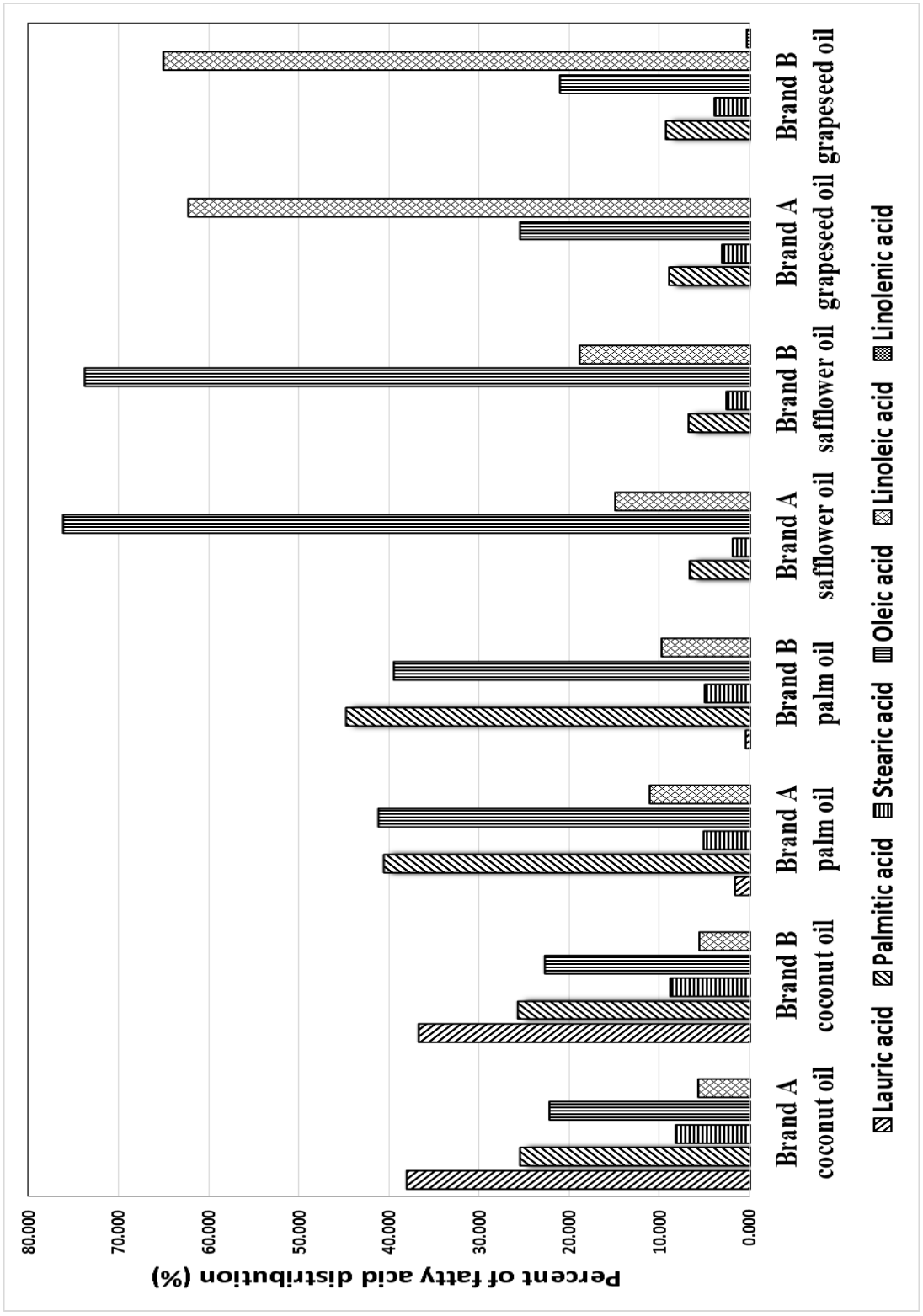

Figure 6: Fatty acid distribution of different vegetable oils

It can be seen clearly in Figure 6 that the linoleic acid in grape seed oil had the highest concentration in unheated oil followed by safflower oil, palm oil, and coconut oil. And we can see coconut and palm oil contained very high saturated fatty acids, while grape seed oil and safflower oil had large amount of unsaturated fatty acids.

4.3 THIOBARBITURIC ACID REACTIVE SUBSTANCES

Table 4-Table 6 and Figure 7 - Figure 10 showed the results of TBARS formation of the 4 oils: coconut oil, palm oil, safflower oil and grape seed oil at 3 different temperatures 165, 185 and 218°C and various times including 0, 0.3, 1, 2, 3, 4, 5 and 6 hours of thermal treatments. 0.3 hour was used to reach the target temperature. TBARS results we measured gave us general information on how the investigated vegetable oils oxidized under heat treatment. The concentration of MDA equivalent was used to express the formation of secondary lipophilic aldehydes and related carbonyl compounds.

Table 4: TBARS results for coconut, palm, safflower and grape seed oil for 0-6 hours heating at 165°C (MDA equivalent (µg/g oil))

Time (h)	brand A coconut oil	brand B coconut oil	brand A palm oil	brand B palm oil	brand A safflower oil	brand B safflower oil	brand A grape seed oil	brand B grape seed oil
0	0.029	0.114	0.130	0.334	0.000	0.339	1.044	0.158
0.3	0.102	0.133	0.592	0.519	0.971	0.723	1.074	0.903
1	0.648	0.379	1.337	1.304	1.018	0.847	1.243	1.684
2	0.695	0.632	1.431	1.391	1.342	1.171	1.435	2.637
3	0.817	0.641	1.768	1.527	1.302	1.374	1.909	3.082
4	0.796	0.800	1.890	1.735	1.606	1.939	2.000	3.300
5	0.840	0.864	2.122	1.894	1.834	1.641	2.839	3.804
6	0.889	0.810	1.639	1.597	1.508	1.349	3.549	2.787

Table 5: TBARS results for coconut, palm, safflower and grape seed oil for 0-6 hours heating at 185°C (MDA equivalent (µg/g oil))

Time (h)	brand A coconut oil	brand B coconut oil	brand A palm oil	brand B palm oil	brand A safflower oil	brand B safflower oil	brand A grape seed oil	brand B grape seed oil
0	0.029	0.114	0.130	0.334	0.000	0.339	1.044	0.158
0.3	0.346	0.362	0.814	1.245	0.468	0.702	1.534	1.820
1	0.683	0.667	1.067	1.592	1.217	0.749	1.609	1.855
2	0.777	0.777	1.447	1.639	1.281	0.782	1.775	1.984
3	0.789	0.913	1.674	1.702	1.592	0.852	1.984	2.047
4	0.814	1.004	2.126	1.913	1.707	1.121	2.117	2.768
5	0.950	1.081	2.033	2.115	1.663	1.276	2.862	3.476
6	0.751	0.950	1.496	1.934	1.463	1.267	2.874	3.598

Table 6: TBARS results for coconut, palm, safflower and grape seed oil for 0-6 hours heating at 218°C (MDA equivalent (µg/g oil)

Time (h)	brand A coconut oil	brand B coconut oil	brand A palm oil	brand B palm oil	brand A safflower oil	brand B safflower oil	brand A grape seed oil	brand B grape seed oil
0	0.029	0.114	0.130	0.334	0.000	0.339	1.044	0.158
0.3	0.409	0.775	1.238	1.304	0.847	0.704	1.065	1.393
1	0.835	0.985	1.627	1.360	1.063	1.060	1.808	2.166
2	0.854	0.978	1.749	1.459	1.419	1.302	1.981	2.443
3	0.995	1.077	1.775	1.733	2.108	1.604	2.351	2.569
4	1.030	1.262	1.953	1.393	2.201	1.883	2.581	2.958
5	0.957	0.831	1.859	1.421	2.098	2.356	2.874	4.055
6	1.074	1.004	1.688	1.124	1.761	2.246	2.993	3.663

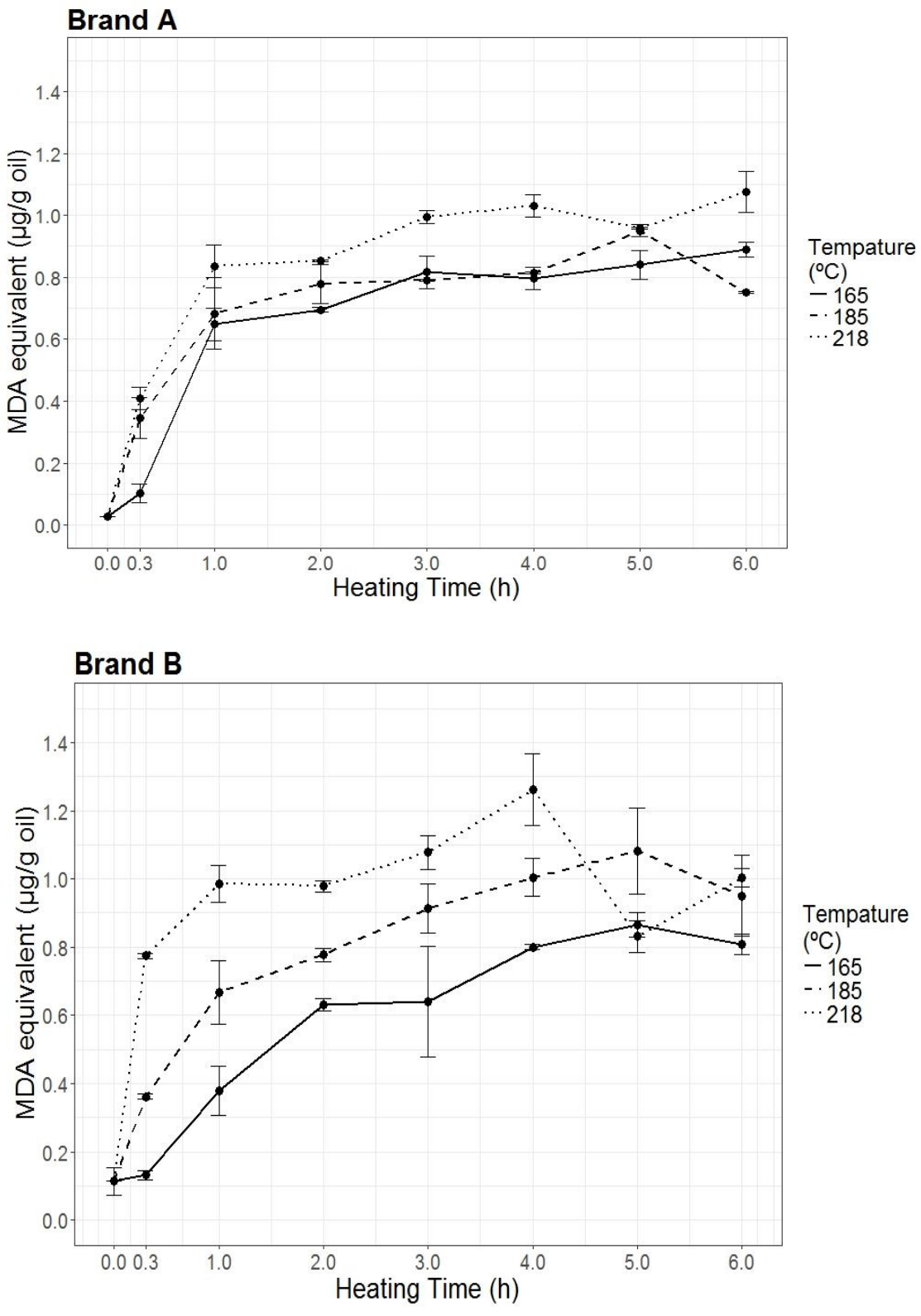

Figure 7: TBARS results for brand A and B coconut oil heating for 0-6h at 165, 185 and 218°C

Figure 7 showed the TBARS results of brand A and B coconut oil heating at 165, 185 and 218°C for 0-6 hours. We can see that oxidation rate and MDA equivalents concentration of secondary oxidation products were the slowest and lowest when heating at 165°C comparing with heating at 185°C, and TBARS results were shown the highest when heating at 218°C. Because coconut oil contained high concentration of saturated fatty acid, such as lauric acid, palmitic acid and stearic acid, the formation of secondary oxidation products was low at all three temperatures. Moreover, the TBARS results from brand A and brand B coconut oil had similar pattern, which was as expected.

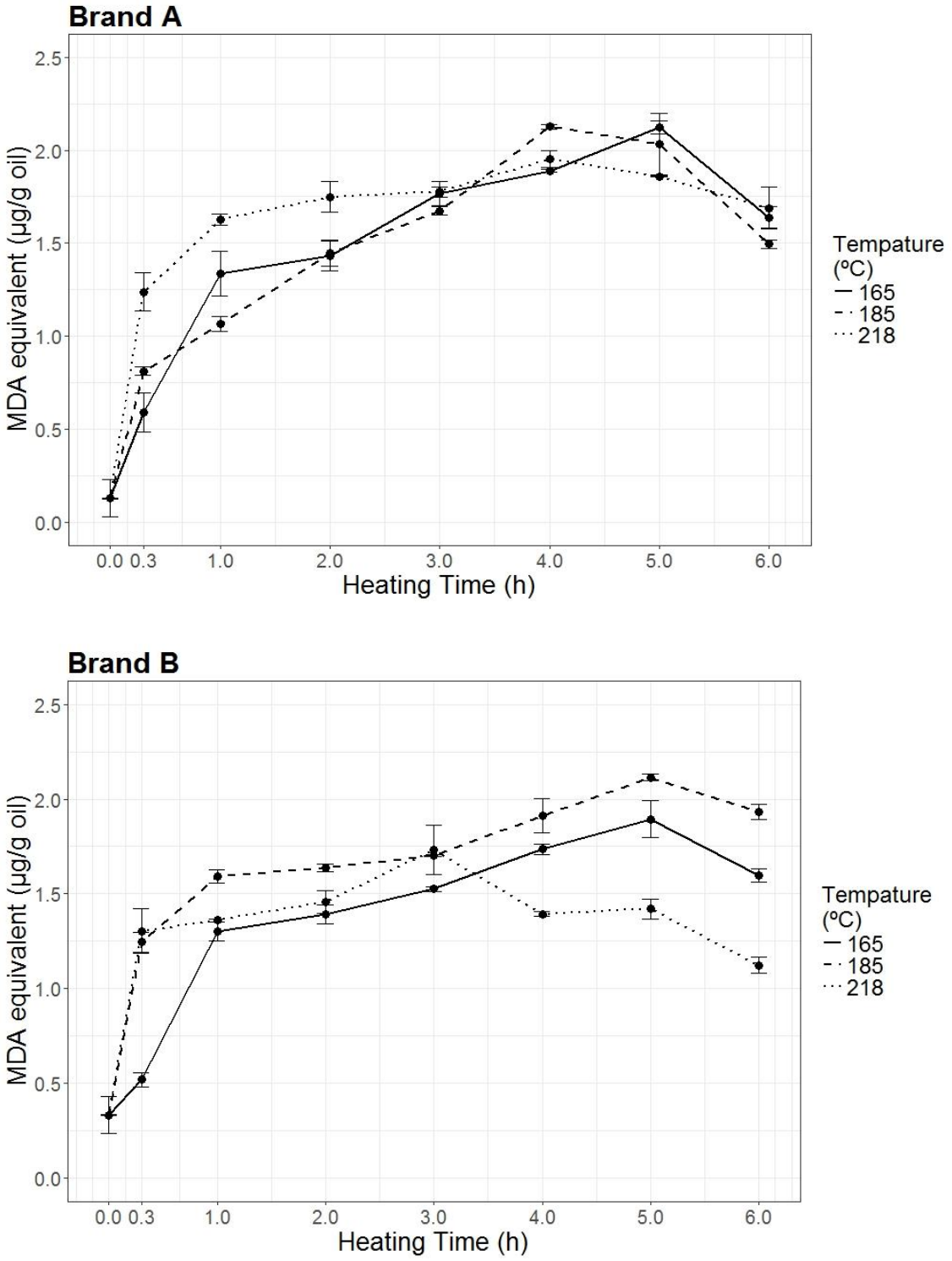

Figure 8: TBARS results for brand A&B palm oil heating for 0-6h at 165, 185 and 218°C

Figure8 presented the TBARS results of brand A and B palm oil heating at 165, 185 and 218°C for 0-6 hours. We can find that palm oil contained less saturated fatty acids than coconut oil; therefore, higher secondary oxidation products were formed. After 5 hours, decomposition of secondary oxidation products started. Both brands showed similar trends under same treatment.

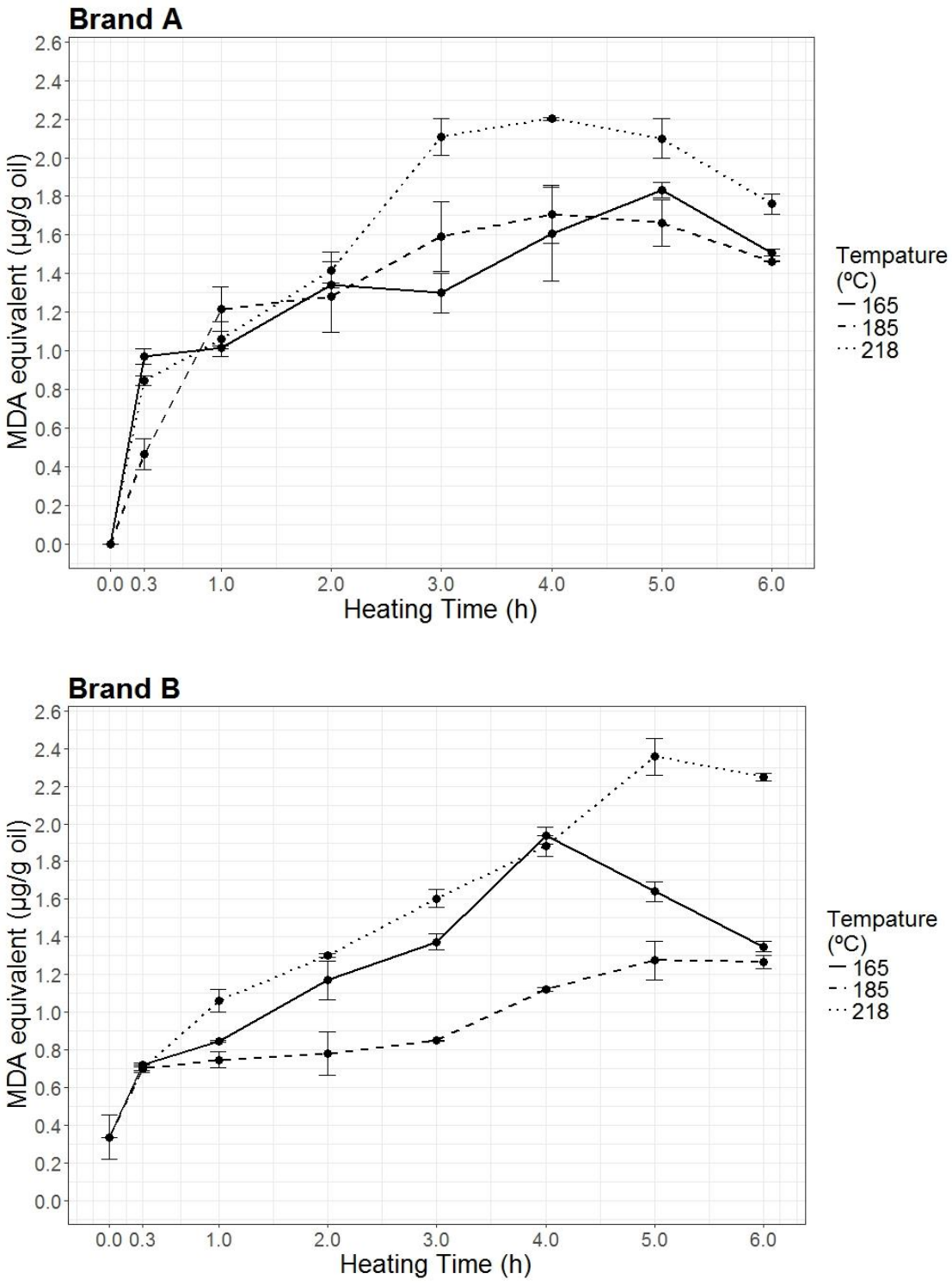

Figure 9: TBARS results for brand A&B safflower oil heating for 0-6h at 165, 185 and 218°

Figure 9 demonstrated the TBARS results of brand A and B safflower oil heating at 165, 185 and 218°C for 0-6 hours. As shown in the figure, oxidation increased rapidly with longer heating time. Because safflower oil was consisted of high concentration of oleic acid, the secondary oxidation products formation was similar to palm oil. And after 5 hours, decomposition of secondary oxidation products began. Both brands showed similar trends under same treatment.

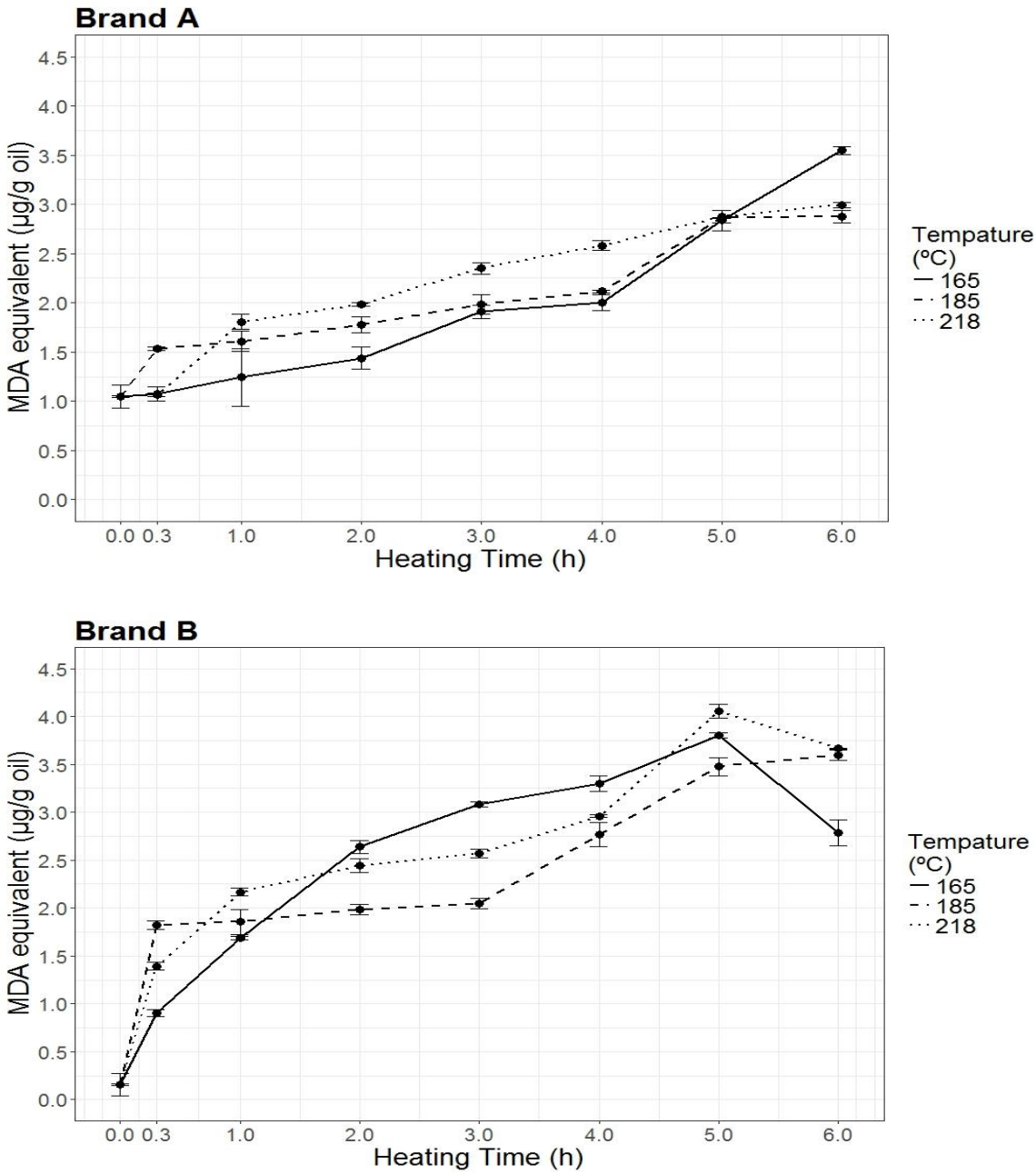

Figure 10: TBARS results for brand A&B grape seed oil heating for 0-6h at 165, 185 and 218°C

Figure 10 demonstrated the TBARS results of brand A and B grape seed oil heating at 165, 185 and 218°C for 0-6 hours. Grape seed oil containing of high linoleic acid showed similar secondary oxidation products formation at all three temperatures.

4.4 TROLOX EQUIVALENT ANTIOXIDANT CAPACITY (TEAC) ASSAY

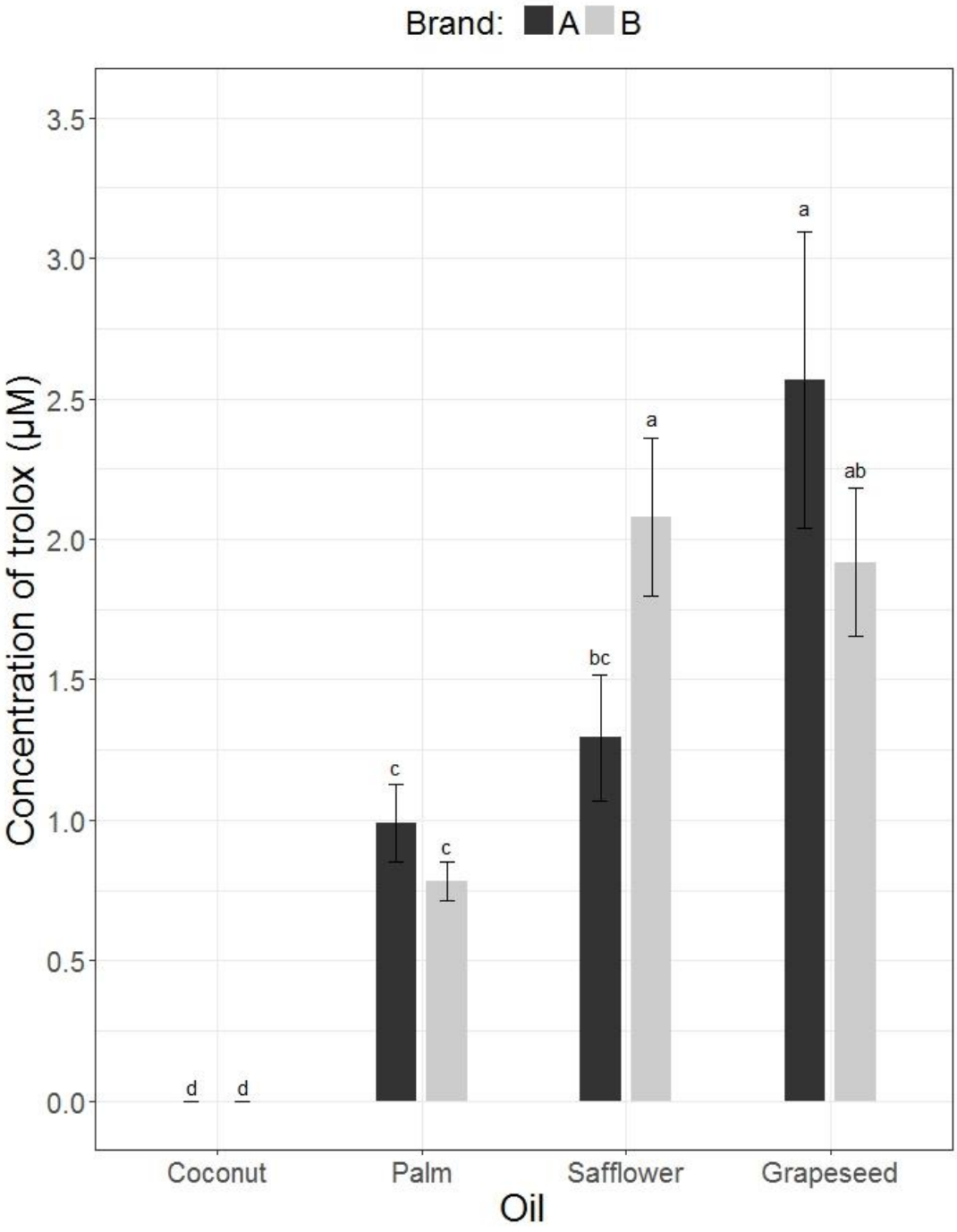

Figure 11: Antioxidant capacity results from Trolox Equivalent Antioxidant Capacity (TEAC) Assay of coconut, grape seed, palm and safflower oil

The TEAC assay provided a reference on the antioxidant capacity of unheated vegetable oils. The bar plot in Figure 11 showed the comparison of antioxidant capacity of coconut, grape seed, palm and safflower oil. We can see clearly from the chart that both brands of coconut oil observed no antioxidant capacity, while other three oils presented antioxidant capacity. Moreover, only the antioxidant capacity of brand A and brand B safflower oil had significant difference. The results from TEAC assay can be used as a factor to explain the influence of antioxidants on secondary oxidation compounds formation.

4.5 Formation of HNE, the Major Toxic a, b-Unsaturated 4-Hydroxyaldehyde, in Commercial Coconut, Palm, Safflower and Grape seed oil under 0, 1, 3 and 5 Hours of Thermal Treatment Heating at 165°C, 185°C and 218 °C

Based on the results from preliminary experiments, the following conditions were selected to measure the formation of HNE under heating temperatures 165, 185 and 218°C and heating time 0, 1, 3 and 5 hours.

ANOVA explanation and results were shown in the appendix. In the ANOVA analysis, temperature, heating time and brand were considered as characters and the response was HNE concentration of investigated oils. The obtained p-values were all compared with significant value 0.05. If p-value is smaller than 0.05, we considered the associated character had significant influence on HNE concentration.

Typical HPLC chromatographs of the four unheated and heated oils, coconut, palm, safflower and grape seed oils were shown from Figure 12-Figure 20.

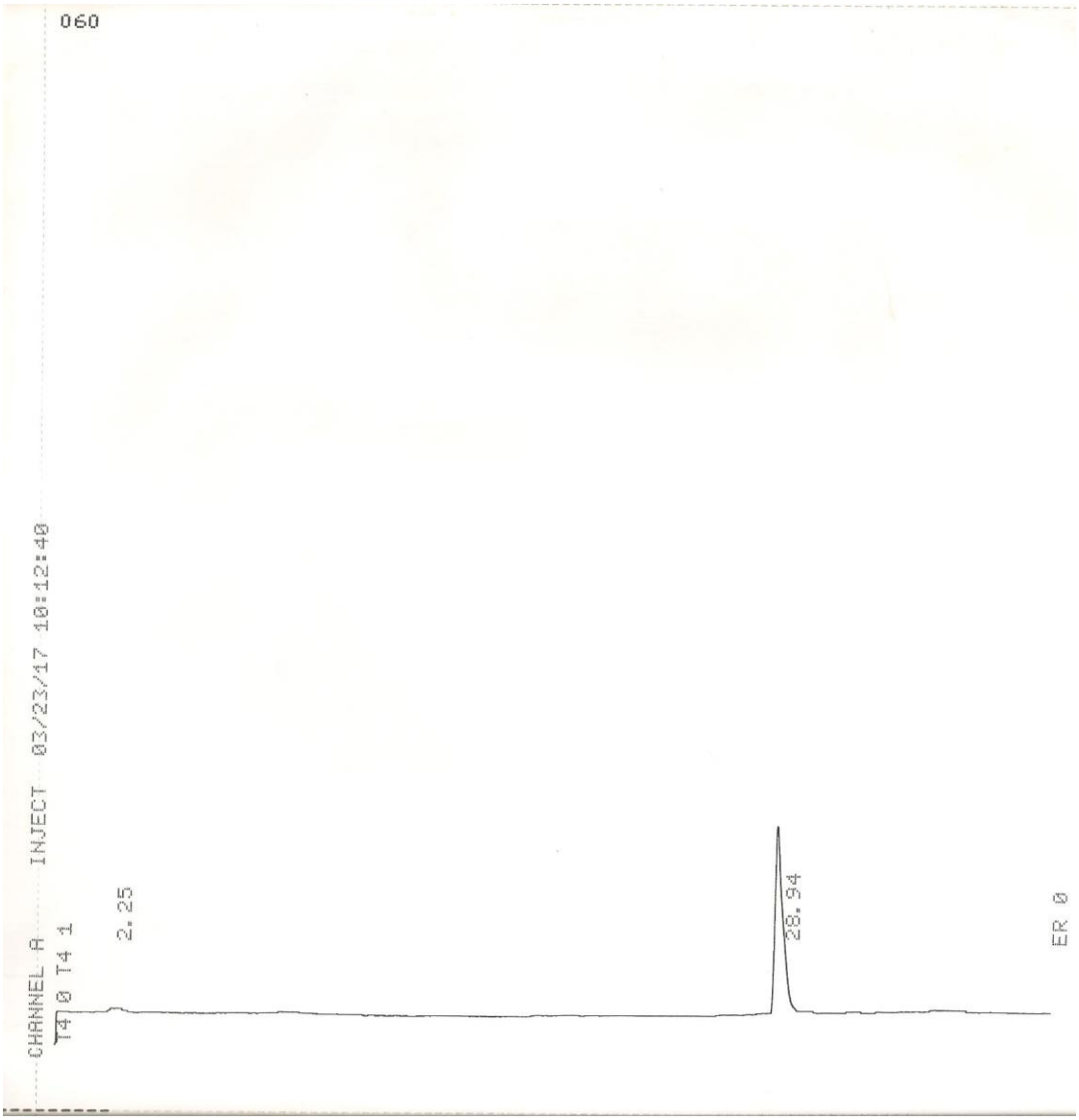

Figure 12: HNE chromatograph of HNE standard

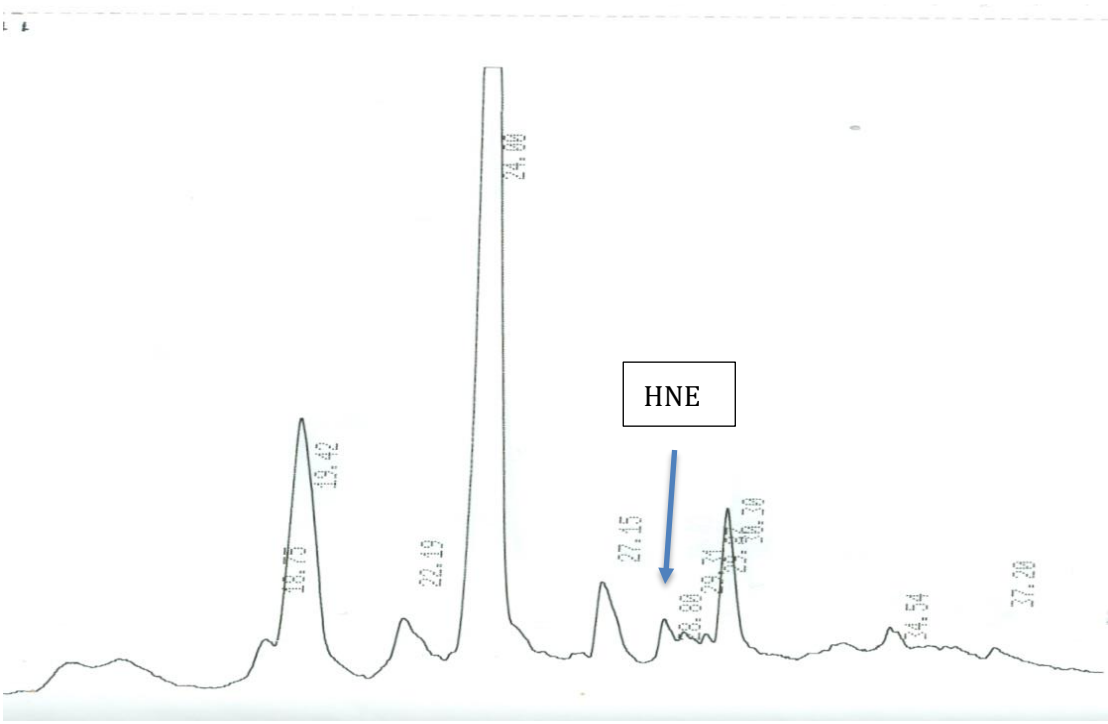

Figure 13: HNE chromatograph of unheated coconut oil, AT=32

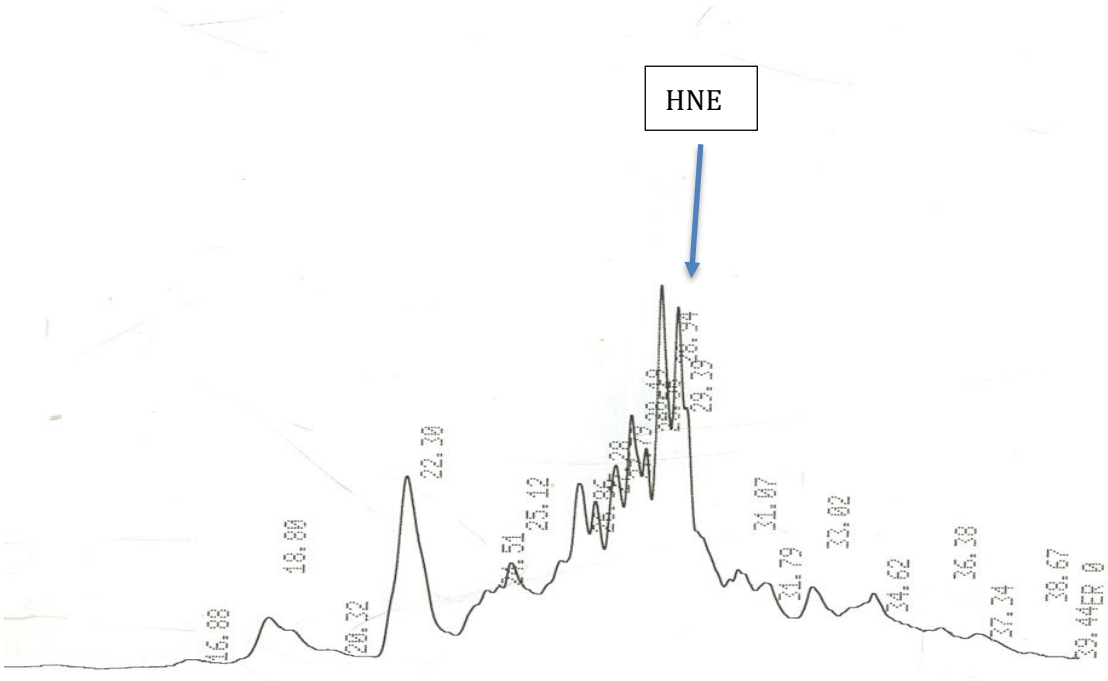

Figure 14: HNE chromatograph of coconut oil heating at 165 ℃ for 1hour, AT=128

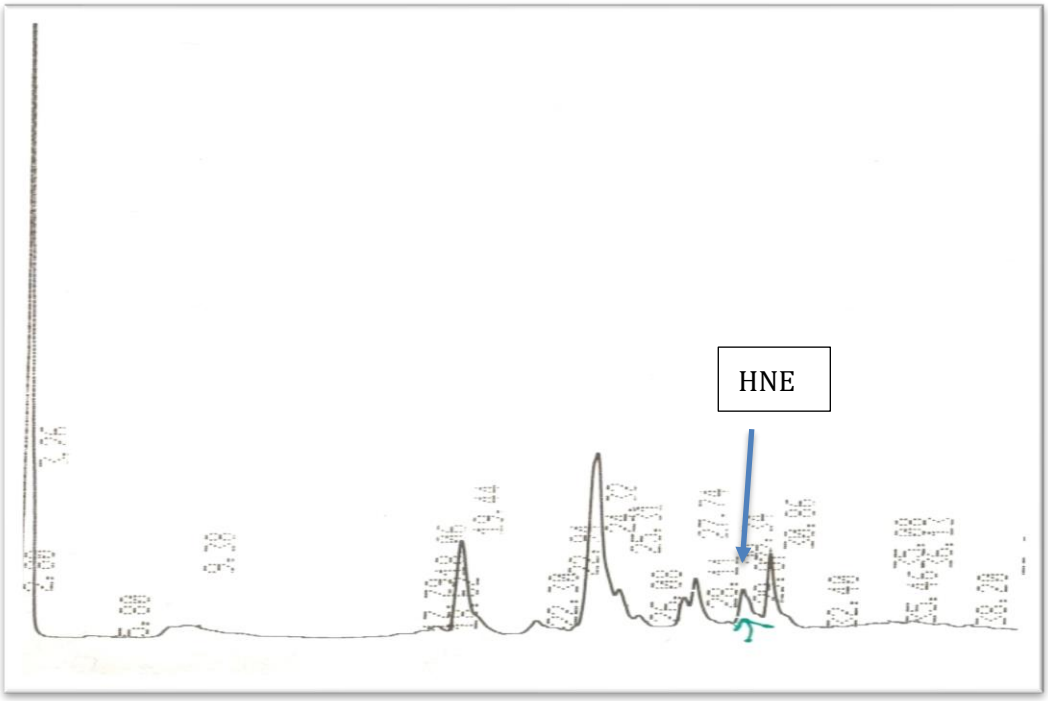

Figure 15: HNE chromatograph of unheated palm oil, AT=128

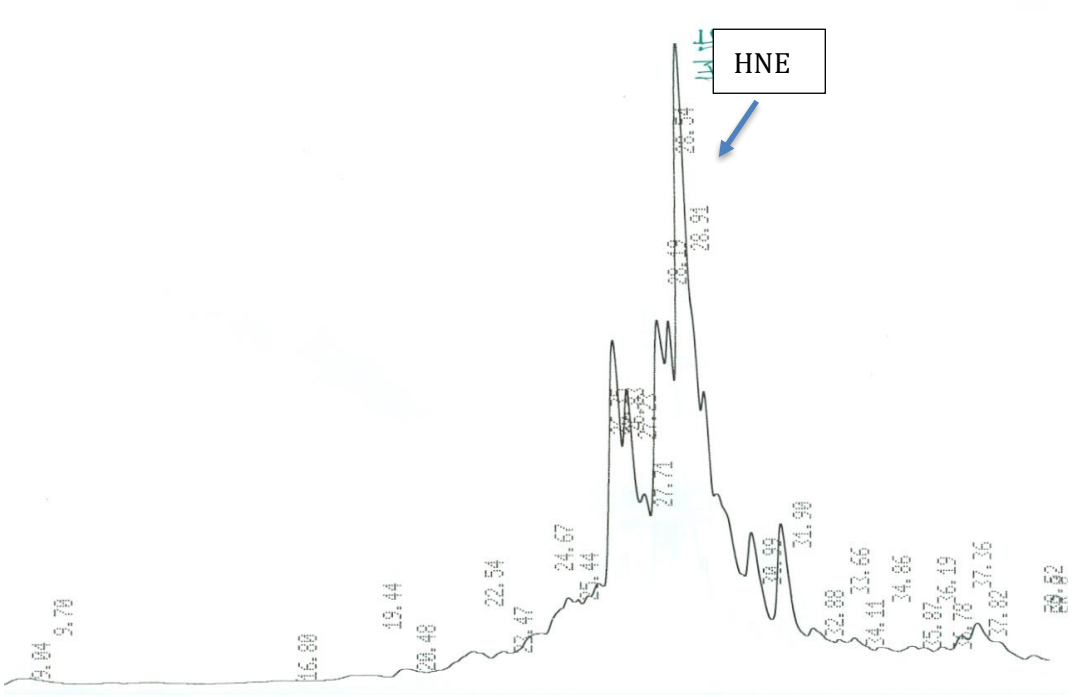

Figure 16: HNE chromatograph of palm oil heating at 165℃ for 3hour, AT=256

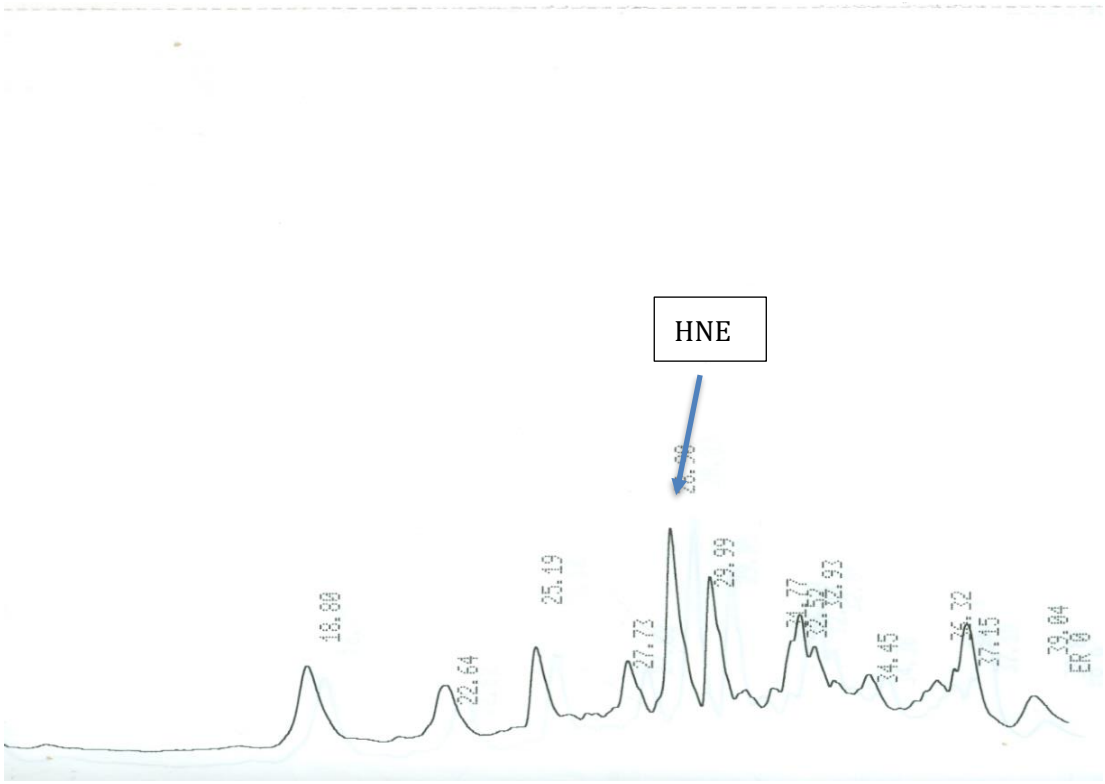

Figure 17: HNE chromatograph of unheated safflower oil, AT=64

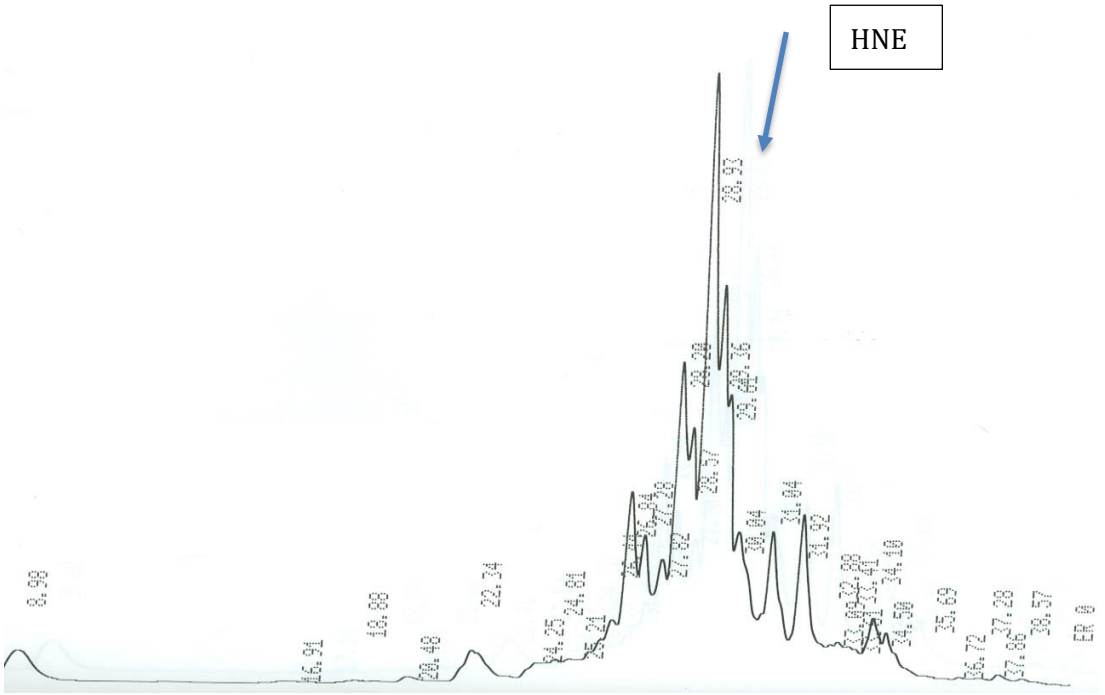

Figure 18: HNE chromatograph of safflower oil heating at 165°C for 5h, AT=512

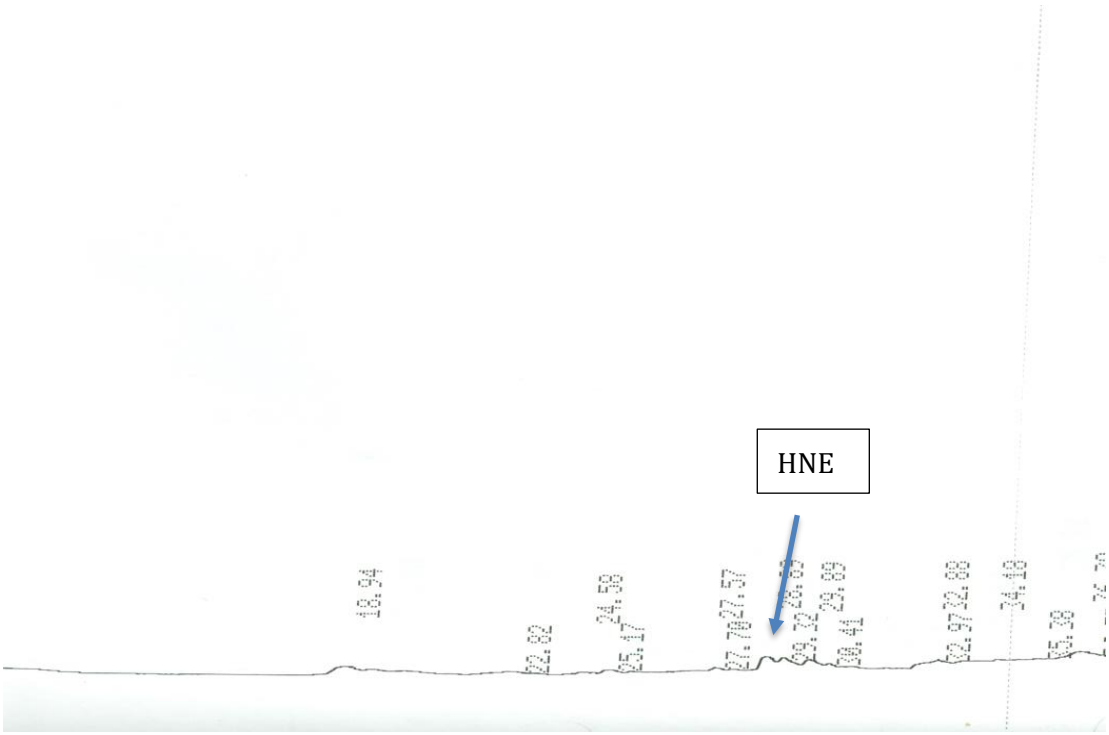

Figure 19: HNE chromatograph of unheated grape seed oil, AT=128

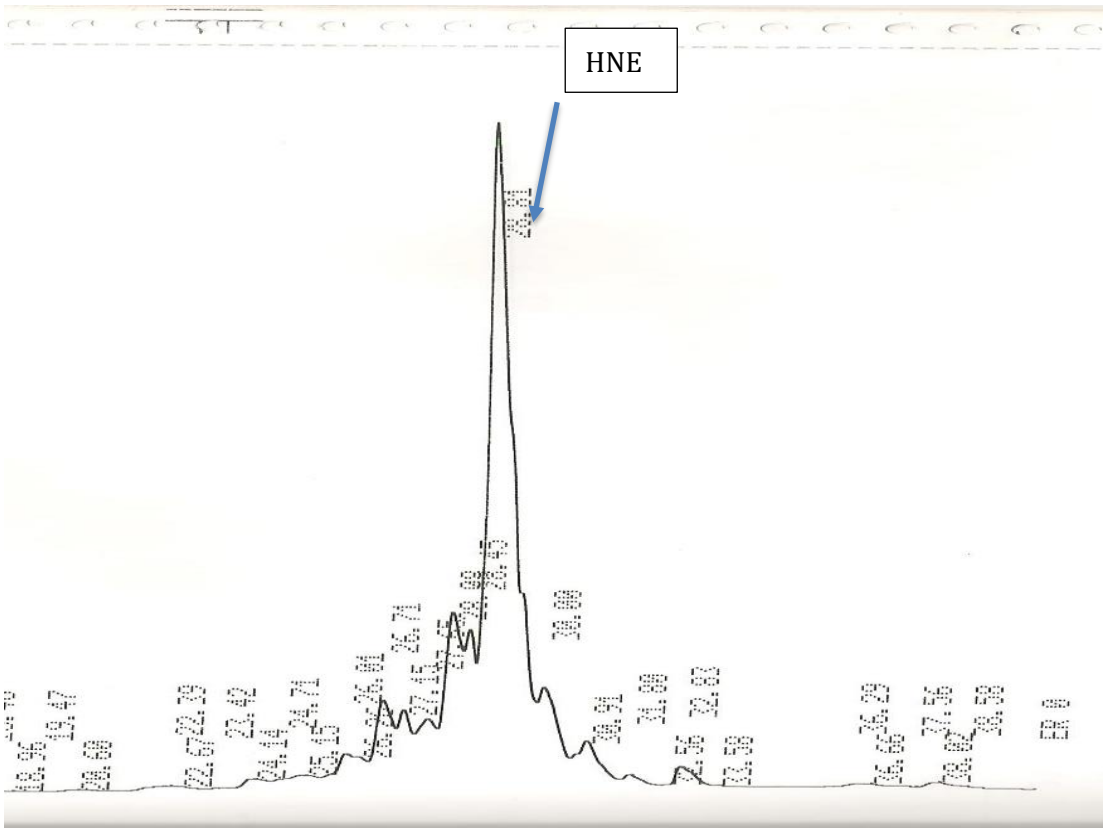

Figure 20: HNE chromatograph of grape seed oil heating at 185℃ for 3h, AT=512

Co-chromatography was conducted on unheated grape seed oil and heated coconut oils. And Table 7 presented co-chromatography results for these oils.

Table 7: Co-chromatography results for heated coconut oils and unheated grape seed oils

	HNE Standard Area	Sample: HNE mixture ratio	Sample Area	Mixture Area	Percent of recovery
Heated brand A coconut oil	1,504, 812	1:1	5,031, 888	3,397,345	103.9%
Heated brand B coconut oil	1,504,812	1:4	5,263,008	2,352,212	95.9%
Unheated brand A grape seed oil	807,955	1:1	303,807	585,898	105.4%
Unheated brand B grape seed oil	807,955	1:1	131,226	483,613	97.1%

a) Coconut oil

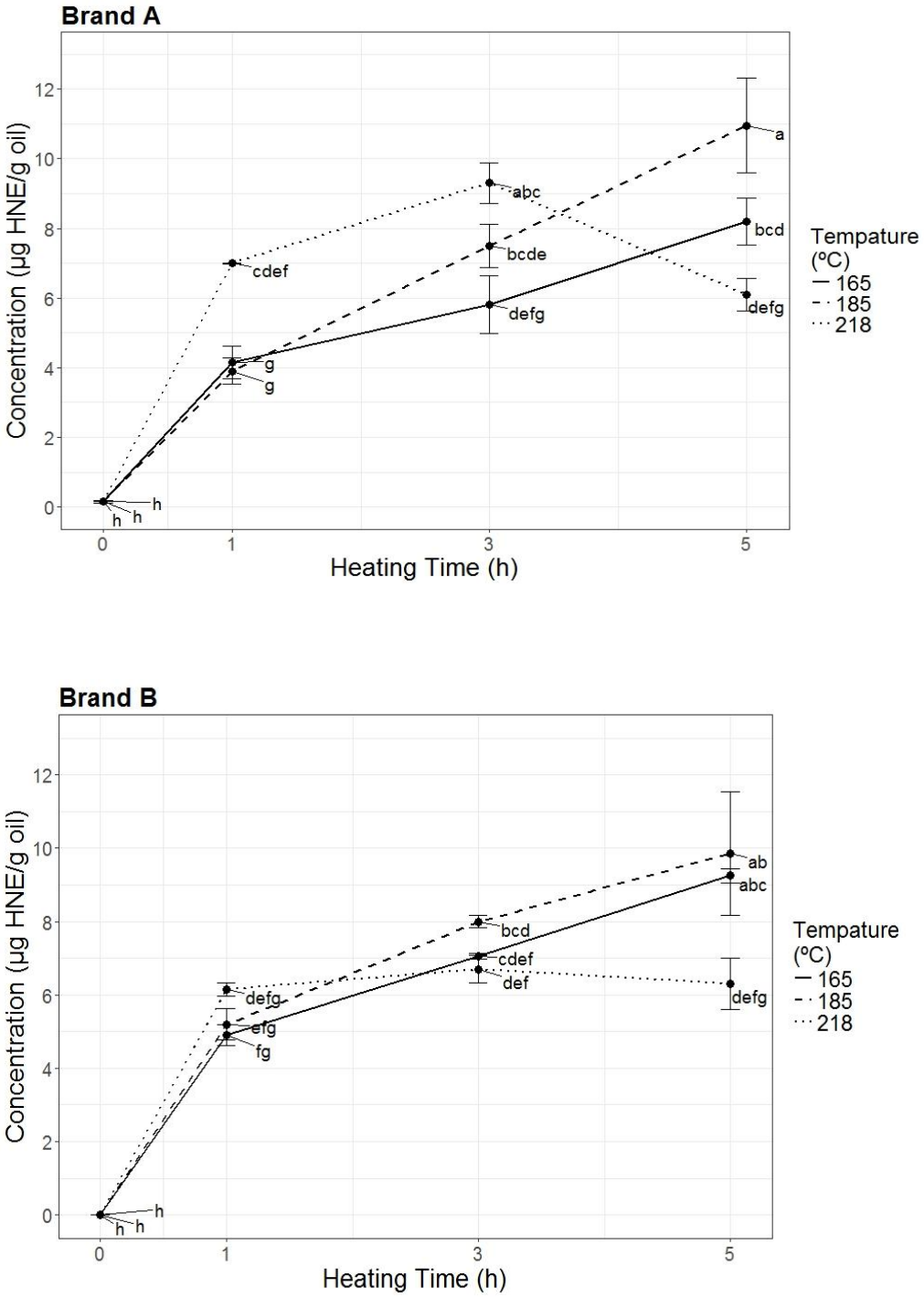

Figure 21: HNE formation in commercial brand A&B coconut oil heated at 165, 185 and 218°C for 0, 1, 3, 5h

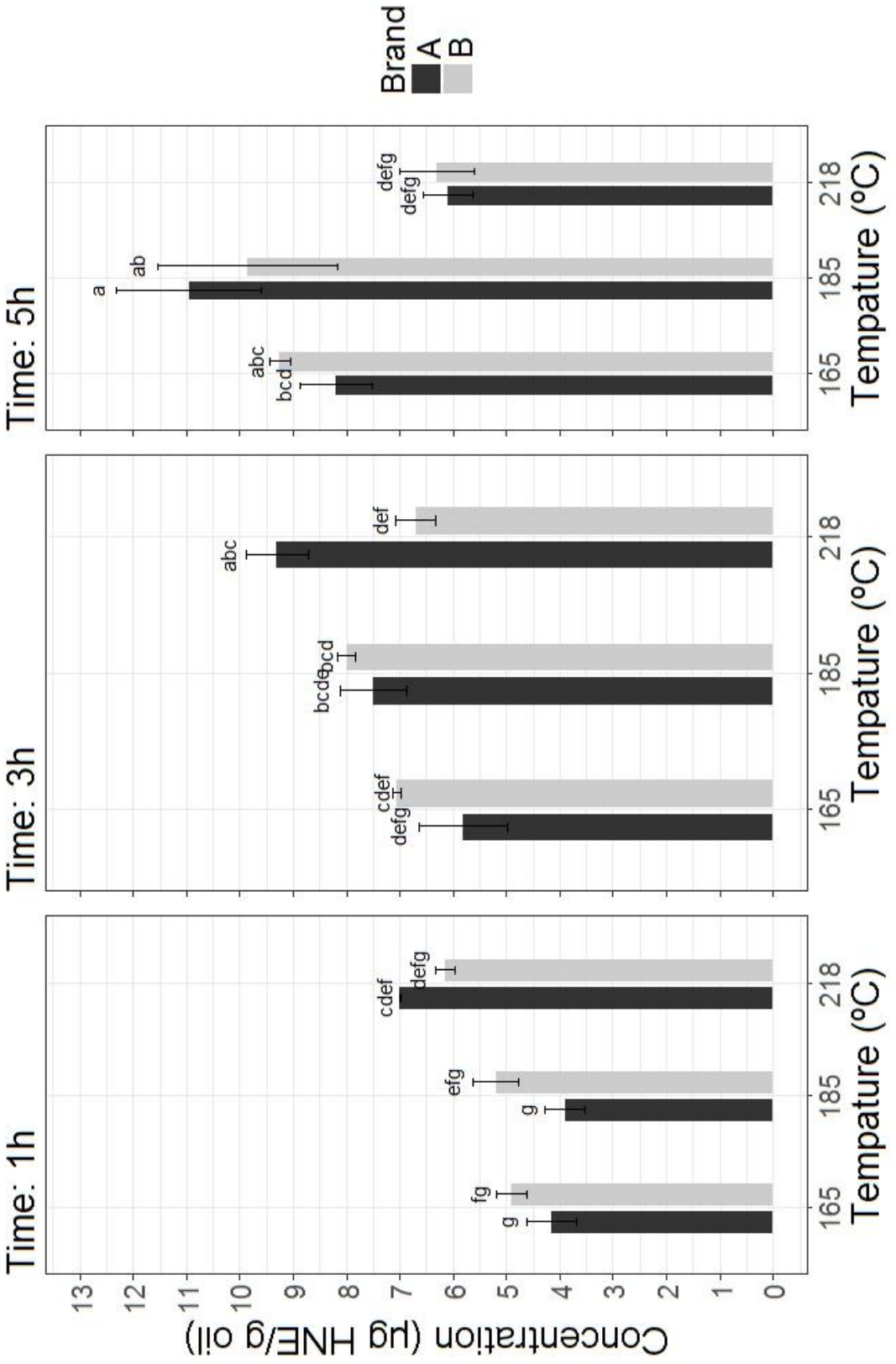

Figure 22: Comparison of HNE formation of coconut oil heating at 165, 185 and 218°C for 1, 3 and 5 hours

Table 8: Average HNE concentration of Brand A&B coconut oil heated at 165, 185 and 218ºC for 0, 1, 3 and 5 hours

	Brand A coconut oil			Brand B coconut oil		
Heating time (h)/ Average HNE concentration (μg/g oil)	165°C	185°C	218°C	165°C	185°C	218°C
0	0.17	0.17	0.17	0.00	0.00	0.00
1	4.13	3.87	6.97	2.58	5.17	6.16
3	4.91	7.48	9.28	4.89	7.07	6.71
5	8.18	10.94	6.14	6.34	9.21	6.27

Error! Reference source not found.-Figure 22 and table 7 presented HNE formation in commercial coconut oil heated at 165, 185 and 218°C for 0, 1, 3, 5h. According to ANOVA results, temperature was found to have significant influence on HNE concentration. It can be seen that when heating at 165°C, HNE formation grew up slowly especially after 1 hour heating. And at 185°C heating temperature, HNE concentration grew up faster than at 165°C heating. When heating at 218°C, we can find that HNE formation increased very fast at the beginning, and after that, it started to decrease due to slow decomposition occurred.

b) Palm oil

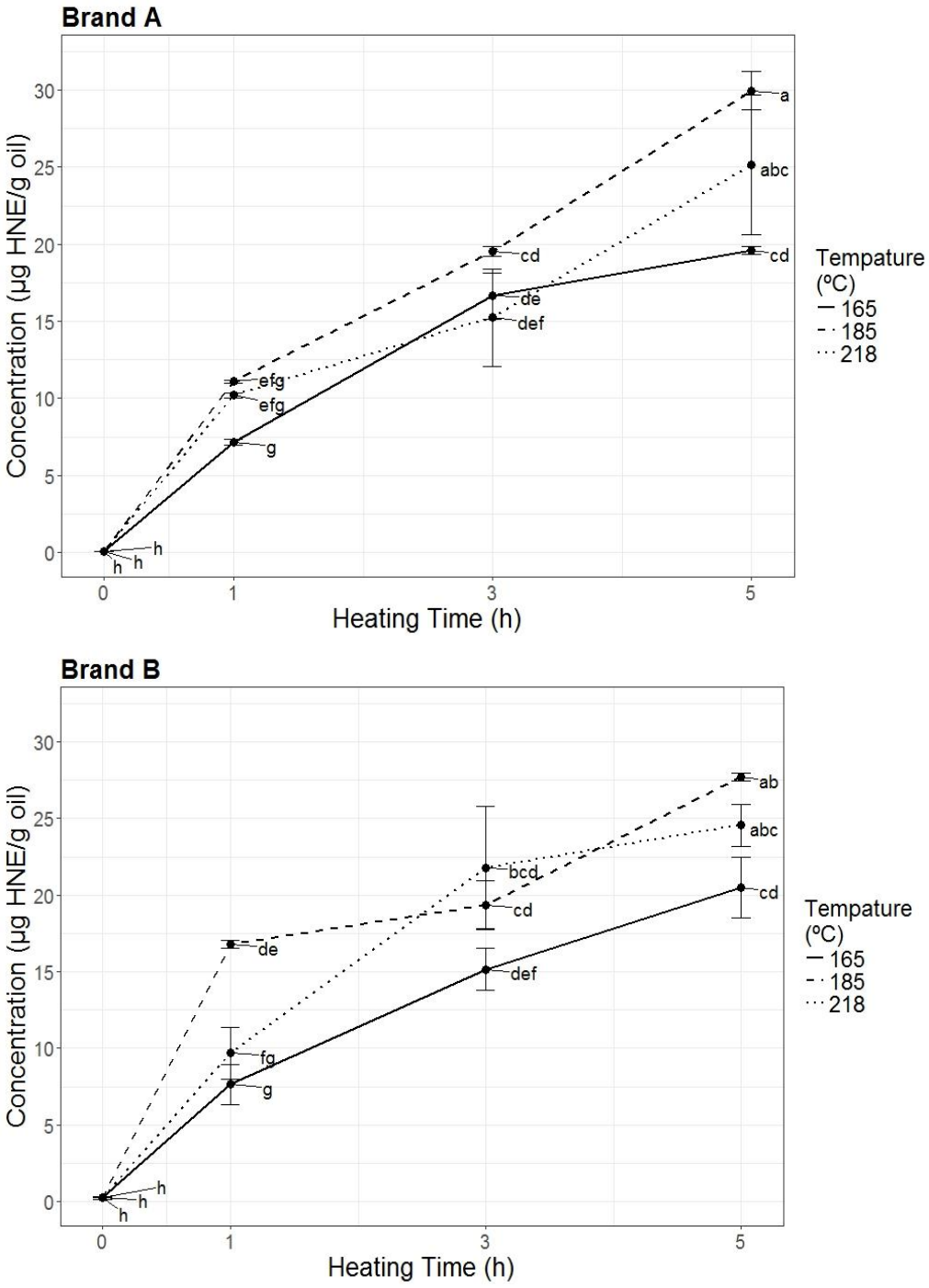

Figure 23: HNE formation in commercial brand A&B palm oil heating at 165, 185 and 218°C for 0, 1, 3, 5h

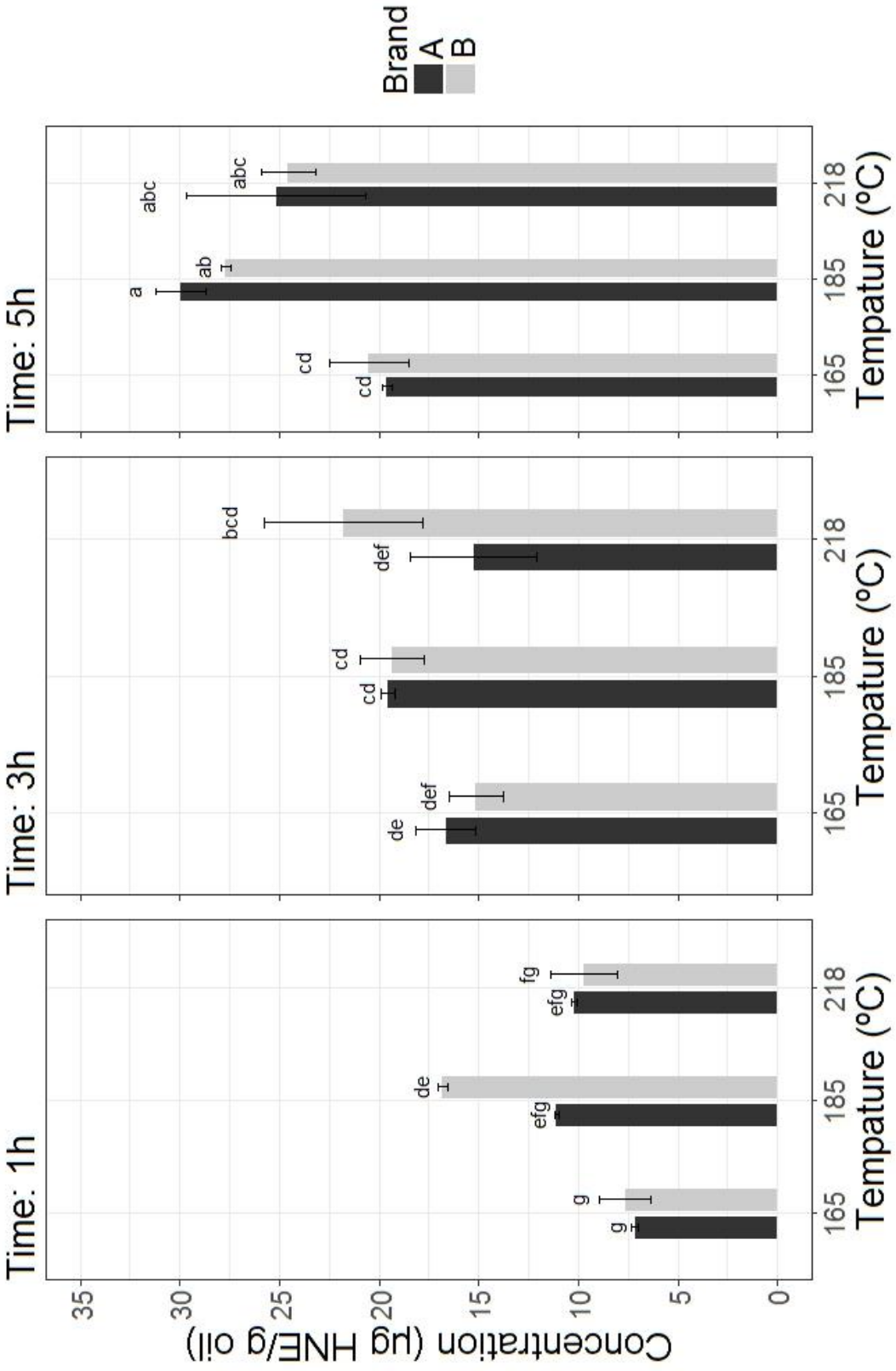

Figure 24: Comparison of HNE formation of palm oil heating at 165, 185 and 218°C for 1, 3 and 5 hours

Table 9: Average HNE concentration of Brand A&B palm oil heating at 165, 185 and 218ºC for 0, 1, 3 and 5 hours

	Brand A palm oil			Brand B palm oil		
Heating time (h)/ Average HNE concentration (µg/g oil)	165°C	185°C	218°C	165°C	185°C	218°C
0	0.08	0.08	0.08	0.25	0.25	0.25
1	7.19	11.10	10.19	7.63	16.77	9.72
3	16.66	19.55	15.23	15.12	19.32	21.81
5	19.63	29.96	25.13	20.48	27.69	24.56

Figure 23-Figure 24 and table 8 demonstrated HNE formation in commercial palm oil heating at 165, 185 and 218°C for 0, 1, 3, 5h. For all three above temperatures, the formation of HNE was shown increased along with heating time. And according to ANOVA results, both temperature and time significantly influenced the formation of HNE. Moreover, as the similar trend was seen in coconut oil, when heating at 165°C, HNE formation grew up more slowly compared to the other two temperatures. At 185°C, HNE concentration showed the highest during 5-hours heating. And when heating at 218°C, two brands of palm oils showed different patterns, but there was no significant difference on HNE concentration within heating periods.

c) Safflower oil

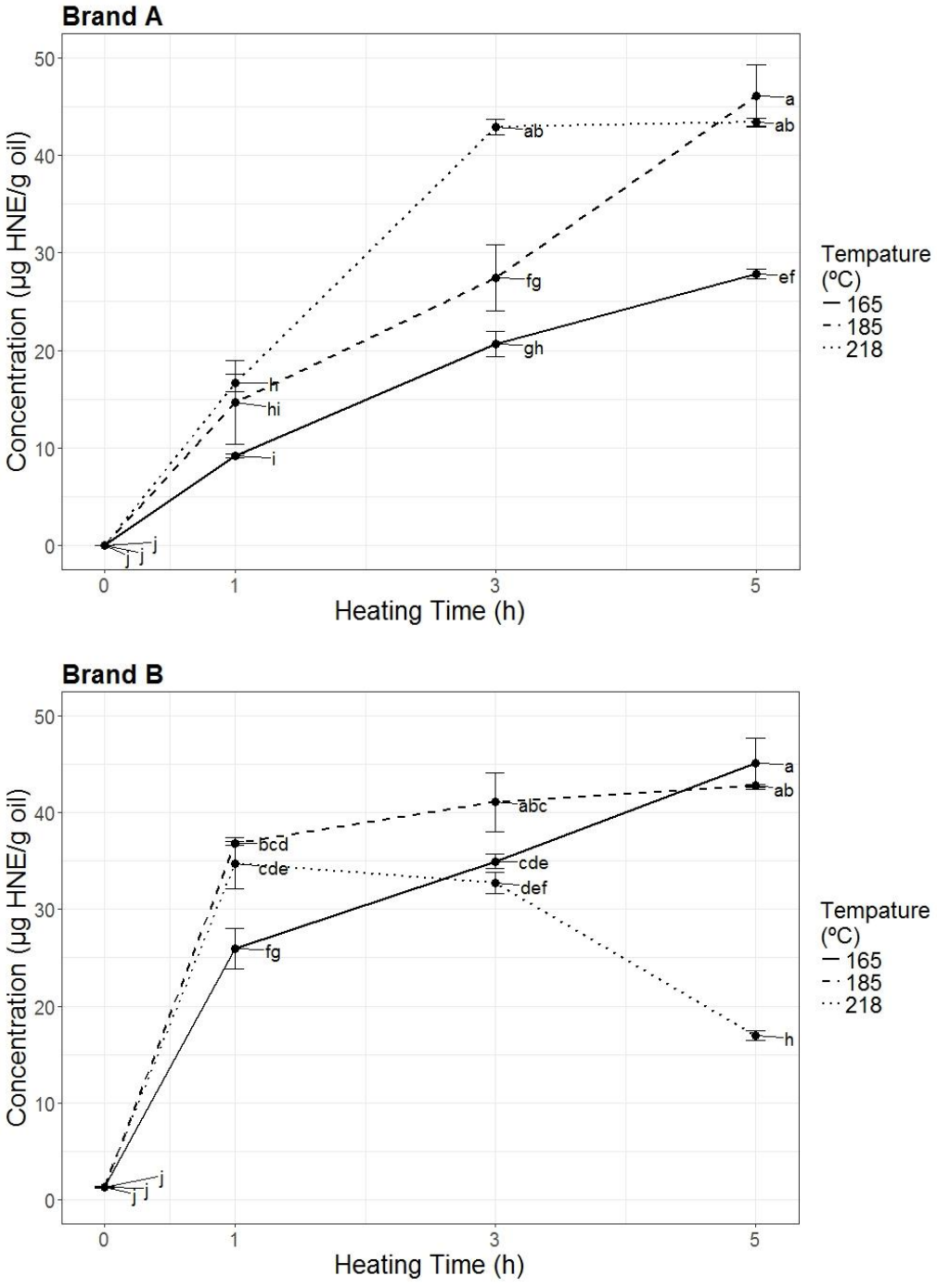

Figure 25: HNE formation in commercial brand A&B safflower oil heating at 165, 185 and 218°C for 0, 1, 3, 5h

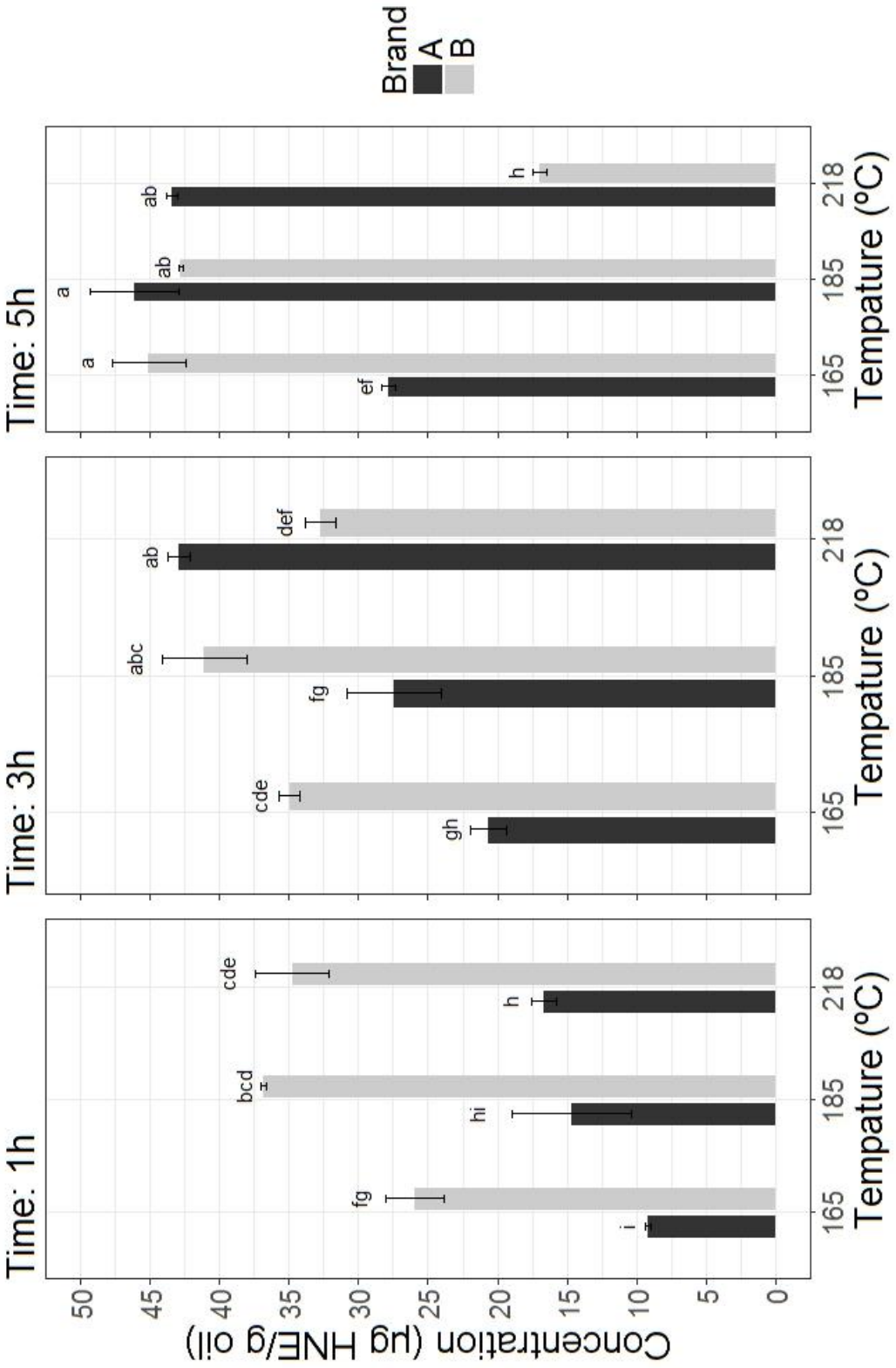

Figure 26: Comparison of HNE formation of safflower oil heating at 165, 185 and 218°C for 1, 3 and 5 hours

Table 10: Average HNE concentration of Brand A&B safflower oil heating at 165, 185 and 218ºC for 0, 1, 3 and 5 hours

	Brand A safflower oil			Brand B safflower oil		
Heating time (h)/ Average HNE concentration (μg/g oil)	165°C	185°C	218°C	165°C	185°C	218°C
0	0.00	0.00	0.00	1.34	1.34	1.34
1	9.21	14.65	16.65	25.96	36.78	34.74
3	20.62	27.46	42.84	34.93	84.17	32.69
5	27.86	46.14	43.40	45.07	48.78	16.95

Figure 25-Figure 26 and table 9 showed HNE formation in commercial safflower oil heating at 165, 185 and 218°C for 0, 1, 3, 5h. No HNE formation was detected in the unheated safflower oil at 0 time of heating. It can be found that HNE formation increased with longer heating time at 165 and 185°C, and HNE concentration was higher when heating at 185°C compared to heating at 165°C. While in terms of 218°C, HNE concentration declined after reaching maximum values. Based on ANOVA analysis, temperature, time and brand all had significant influence on HNE formation.

d) Grape seed oil

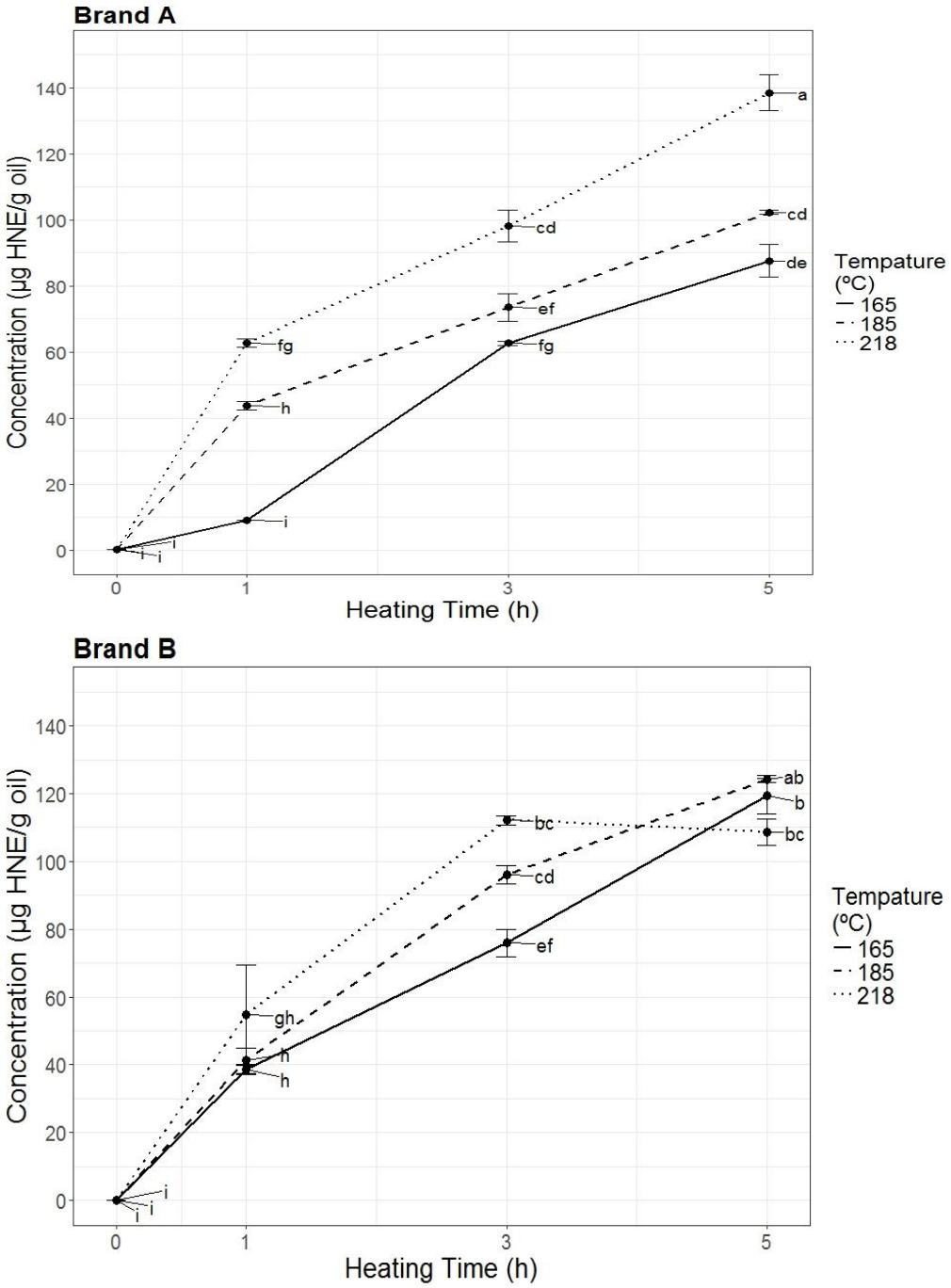

Figure 27: HNE formation in commercial brand A&B grape seed oil heating at 165, 185 and 218°C for 0, 1, 3, 5h

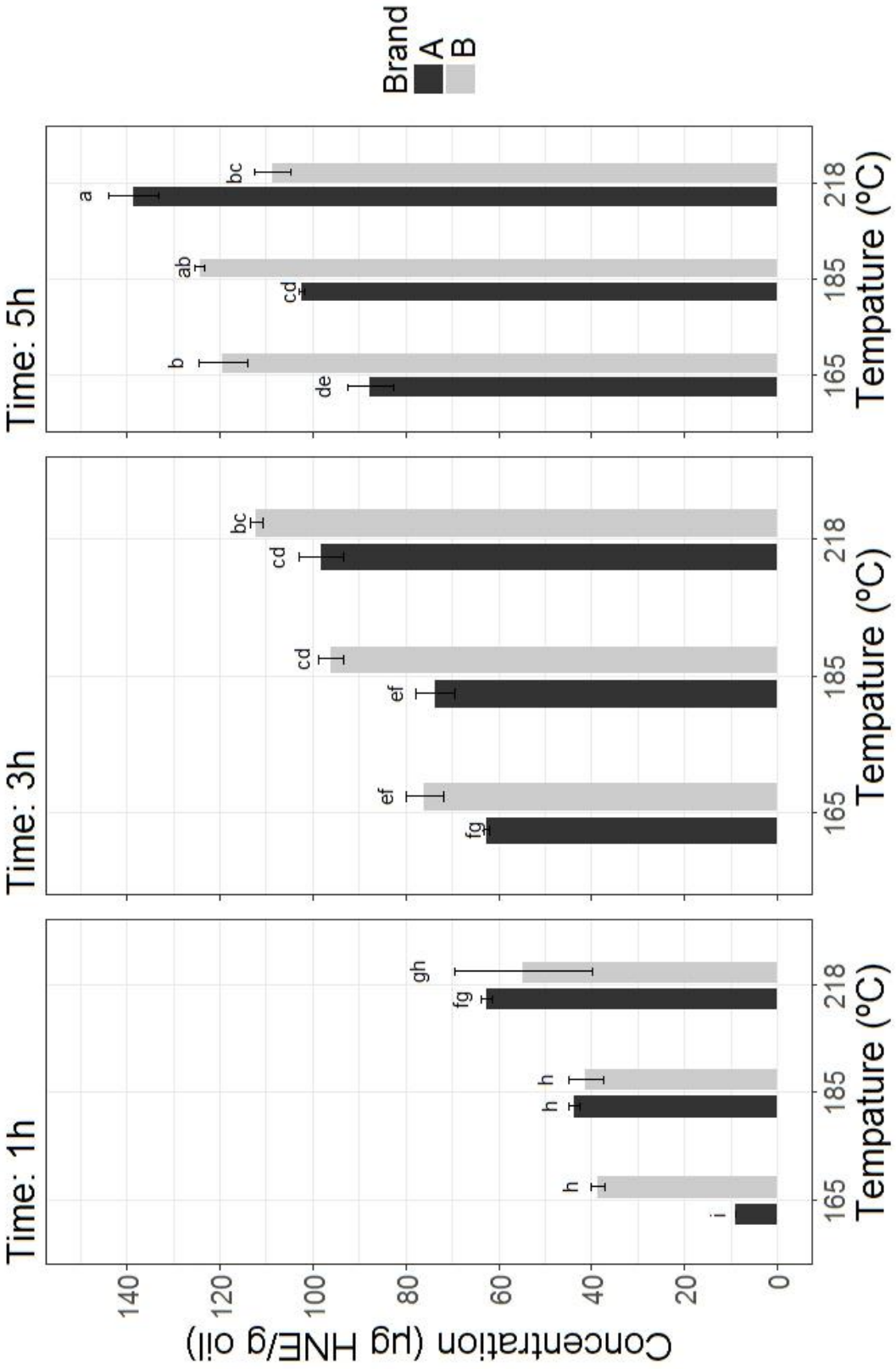

Figure 28: Comparison of HNE formation of grape seed oil heating at 165, 185 and 218°C for 1, 3 and 5 hours

Table 11: Average HNE concentration of Brand A&B grape seed oil heating at 165, 185 and 218ºC for 0, 1, 3 and 5 hours

	Brand A grape seed oil			Brand B grape seed oil		
Heating time (h)/ Average HNE concentration (μg/g oil)	165	185	218	165	185	218
0	0.13	0.13	0.13	0.15	0.15	0.15
1	9.02	43.68	62.66	38.70	41.19	54.61
3	22.66	73.59	98.17	75.89	96.07	112.08
5	87.60	102.33	138.46	119.28	124.30	108.67

Figure 27-Figure 28 and table10 illustrated HNE formation in commercial grape seed oil heating at 165, 185 and 218°C for 0, 1, 3, 5h. We can see that there was an increasing trend on HNE formation with prolonged heat treatment. According to ANOVA analysis on grape seed oil, temperature and time affected significantly on HNE formation. And temperature 218°C induced the highest HNE concentration, followed by 185°C and 165°C. It was obviously to see that in general, at 1 hour heating period, HNE formation increased more slowly when heating at 165°C compared to the other two temperatures. And at 218°C, HNE formation was higher than heating at 185°C.

e) Comparison of HNE concentration in commercial coconut, palm, safflower and grape seed oil heat-treated for 1, 3 and 5 hours at 165, 185 and 218°C

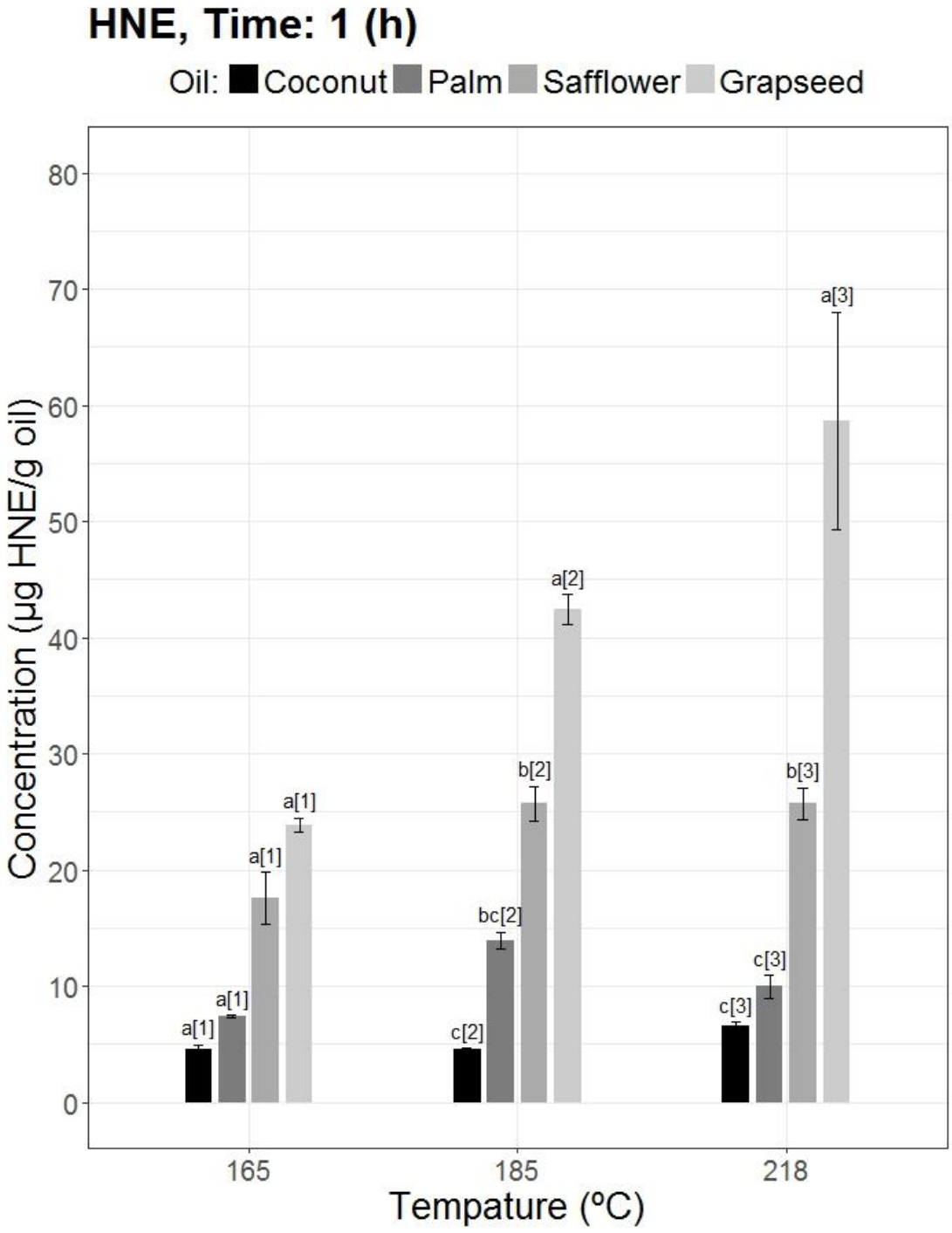

Figure 29: Comparison of average HNE concentrations of two brands of coconut, palm, safflower and grape seed oils heating at 165°C, 185°C and 218°C for 1 hours

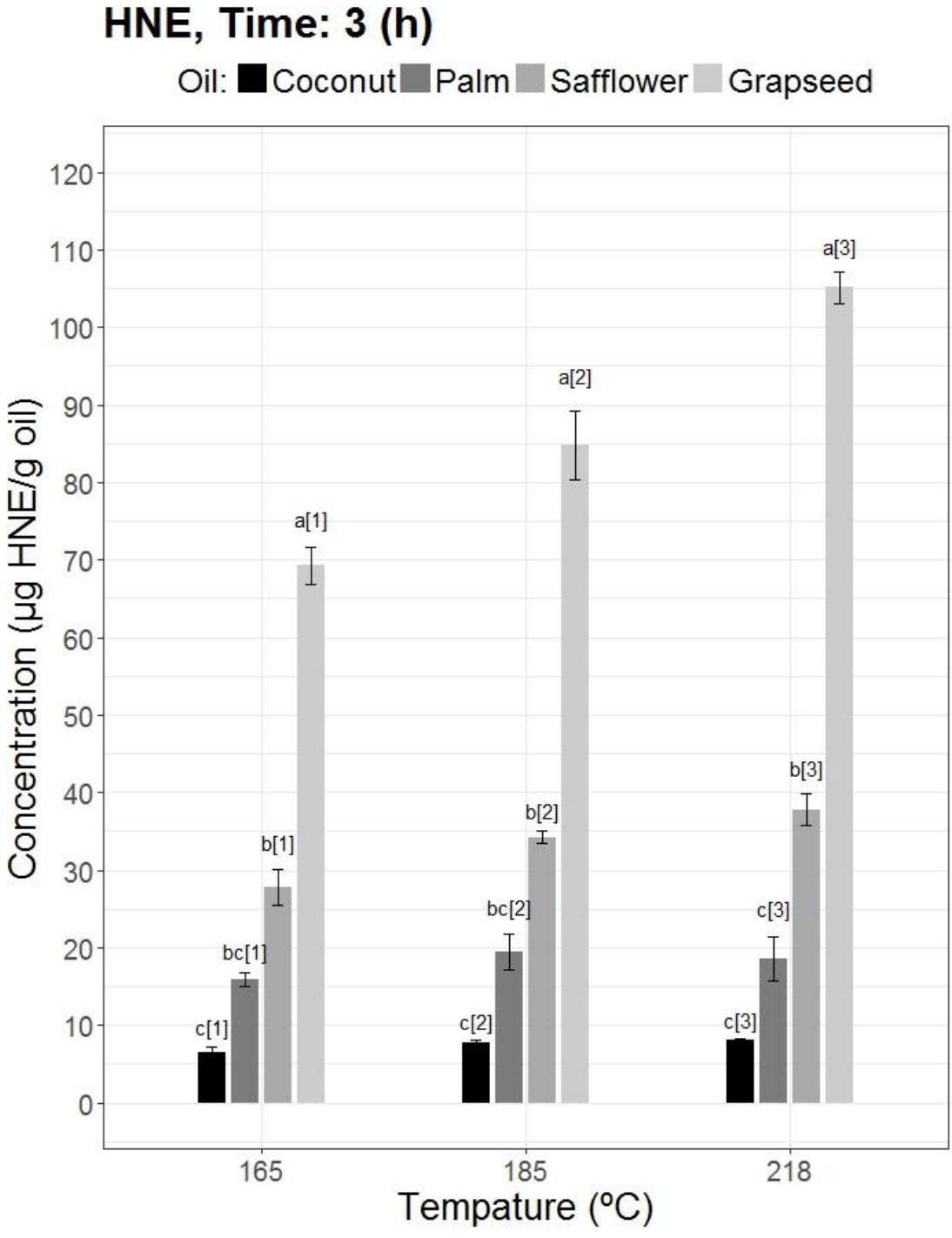

Figure 30: Comparison of average HNE concentration of two brands of coconut, palm, safflower and grape seed oils heating at 165°C, 185°C and 218°C for 3 hours

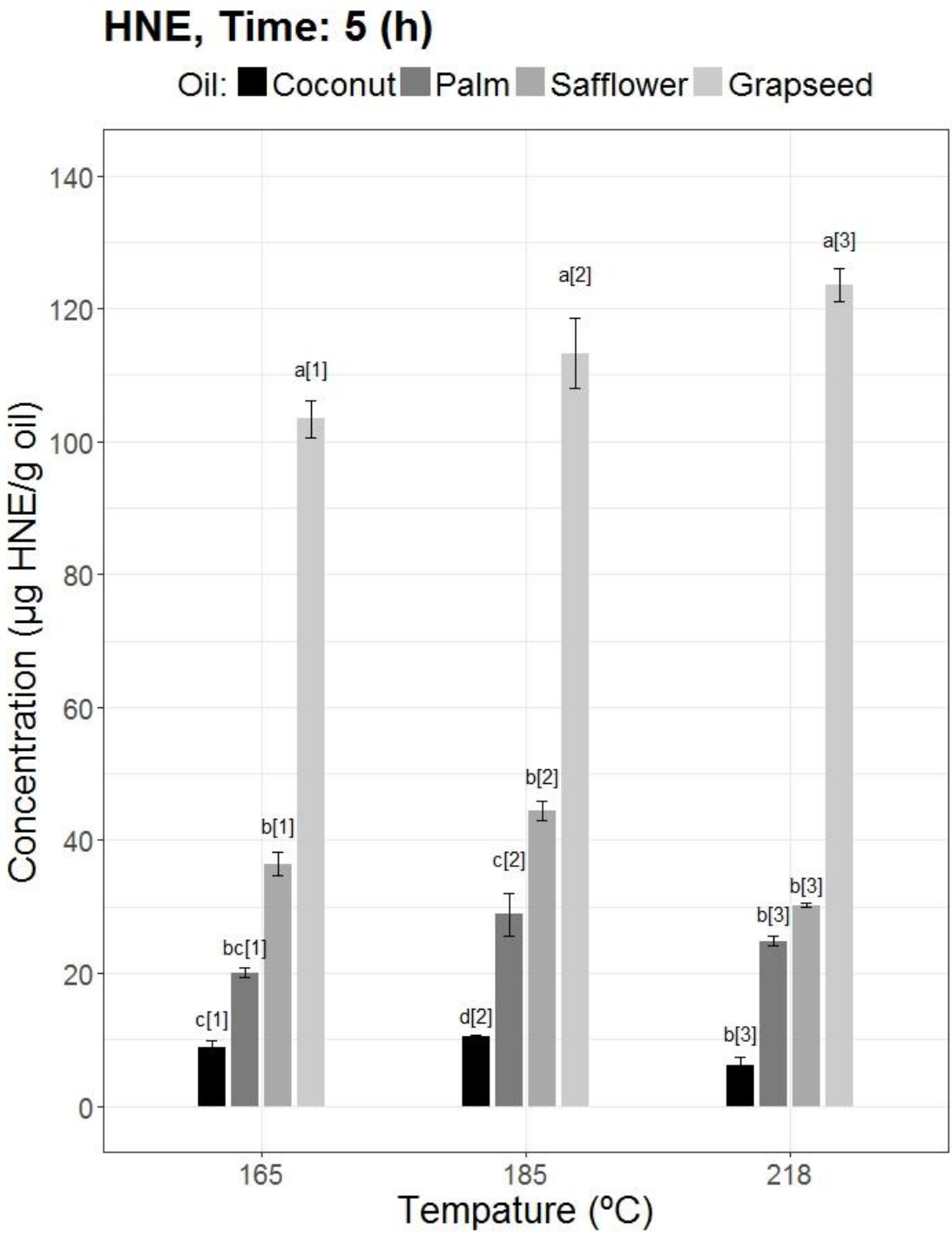

Figure 31: Comparison of average HNE concentration of two brands of coconut, palm, safflower and grape seed oils heating at 165°C, 185°C and 218°C for 5 hours

Figure 29-Figure 31 illustrated the average HNE concentrations of two brands of four vegetable oils heated at 165, 185 and 218°C for 1, 3 and 5 hours. By comparing the temperature at 165°C and 185°C, it can be seen that HNE concentration in four oils increased with higher heating temperature during the same heating periods. In general, the formation rate of HNE was higher at 185°C compared to at 165°C heating. Heating at 218°C, some decomposition occurred in coconut, palm and safflower oils, except in grape seed oils.

4.6 THE FORMATION OF THE SUM TOTAL OF INDIVIDUAL POLAR LIPOPHILIC ALDEHYDES IN COMMERCIAL COCONUT, PALM, SAFFLOWER AND GRAPE SEED OIL HEAT TREATED FOR UP TO 5 HOURS AT 165, 185 AND 218°C

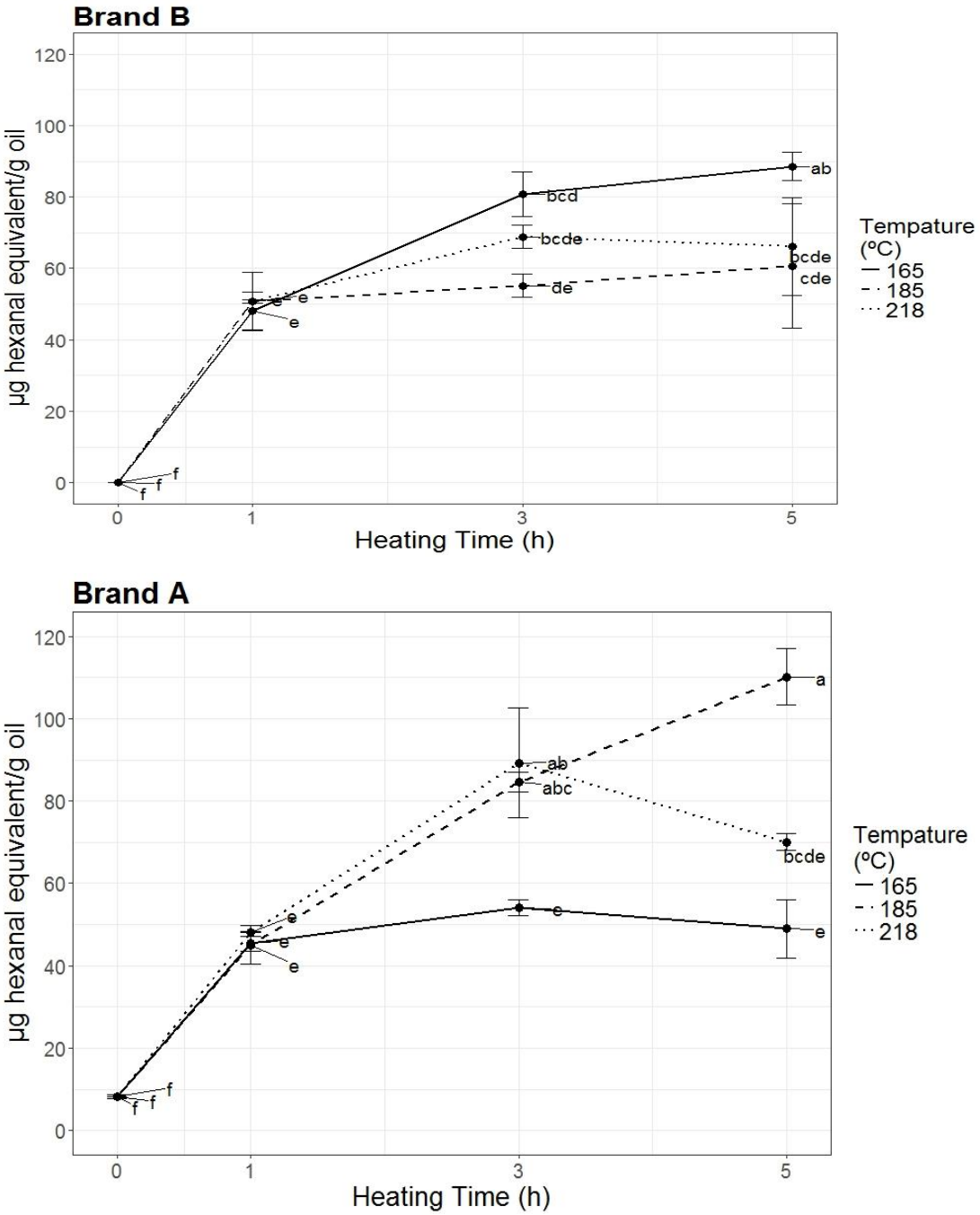

Figure 32: Formation of the sum total of polar lipophilic aldehydes in commercial brand A&B coconut oil heat treated for up to 5 hours at 165, 185 and 218°C

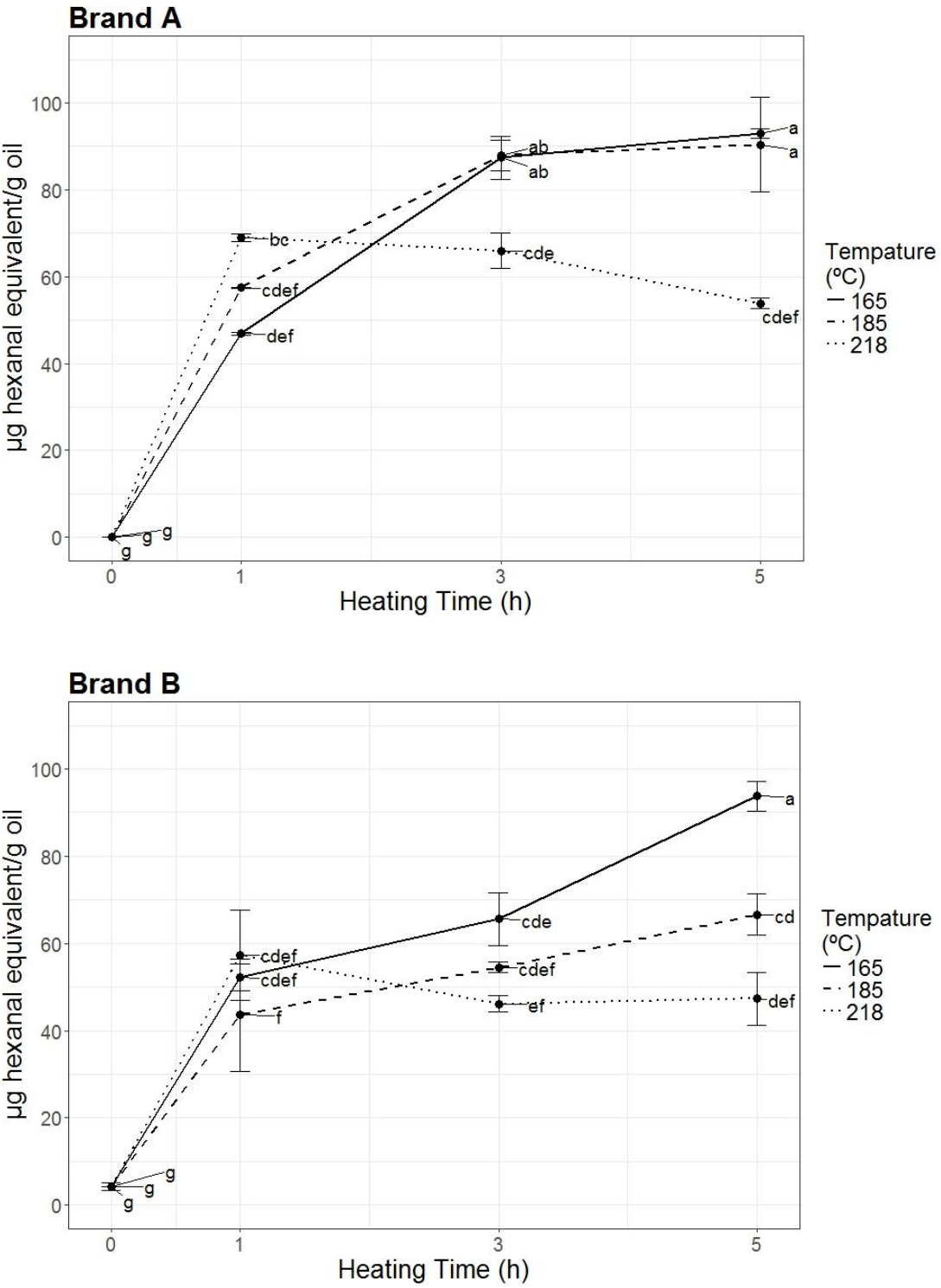

Figure 33: Formation of the sum total of polar lipophilic aldehydes in commercial brand A&B palm oil heat treated for up to 5 hours at 165, 185 and 218°C

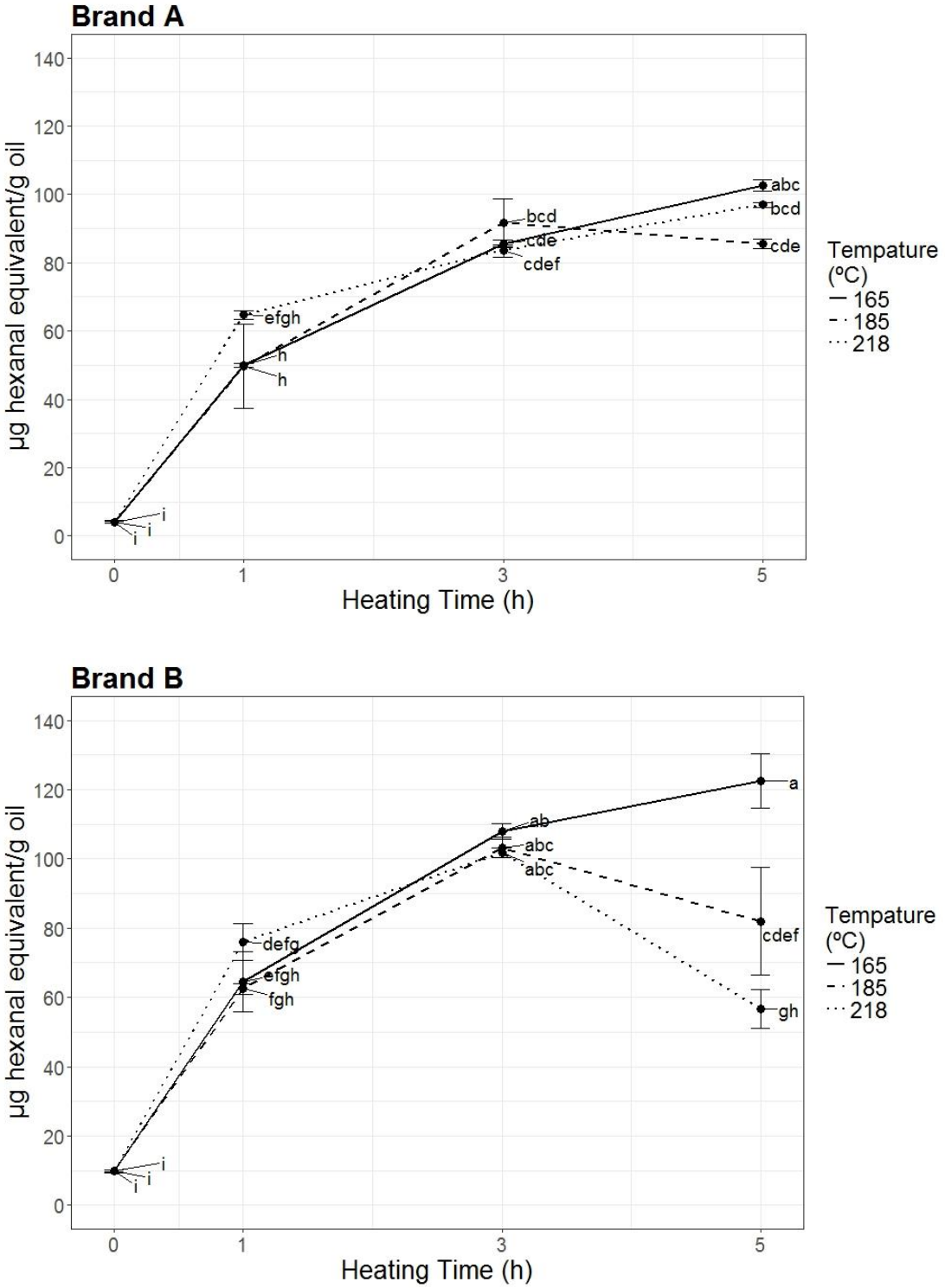

Figure 34: Formation of the sum total of polar lipophilic aldehydes in commercial brand A&B safflower oil heat treated for up to 5 hours at 165, 185 and 218°C

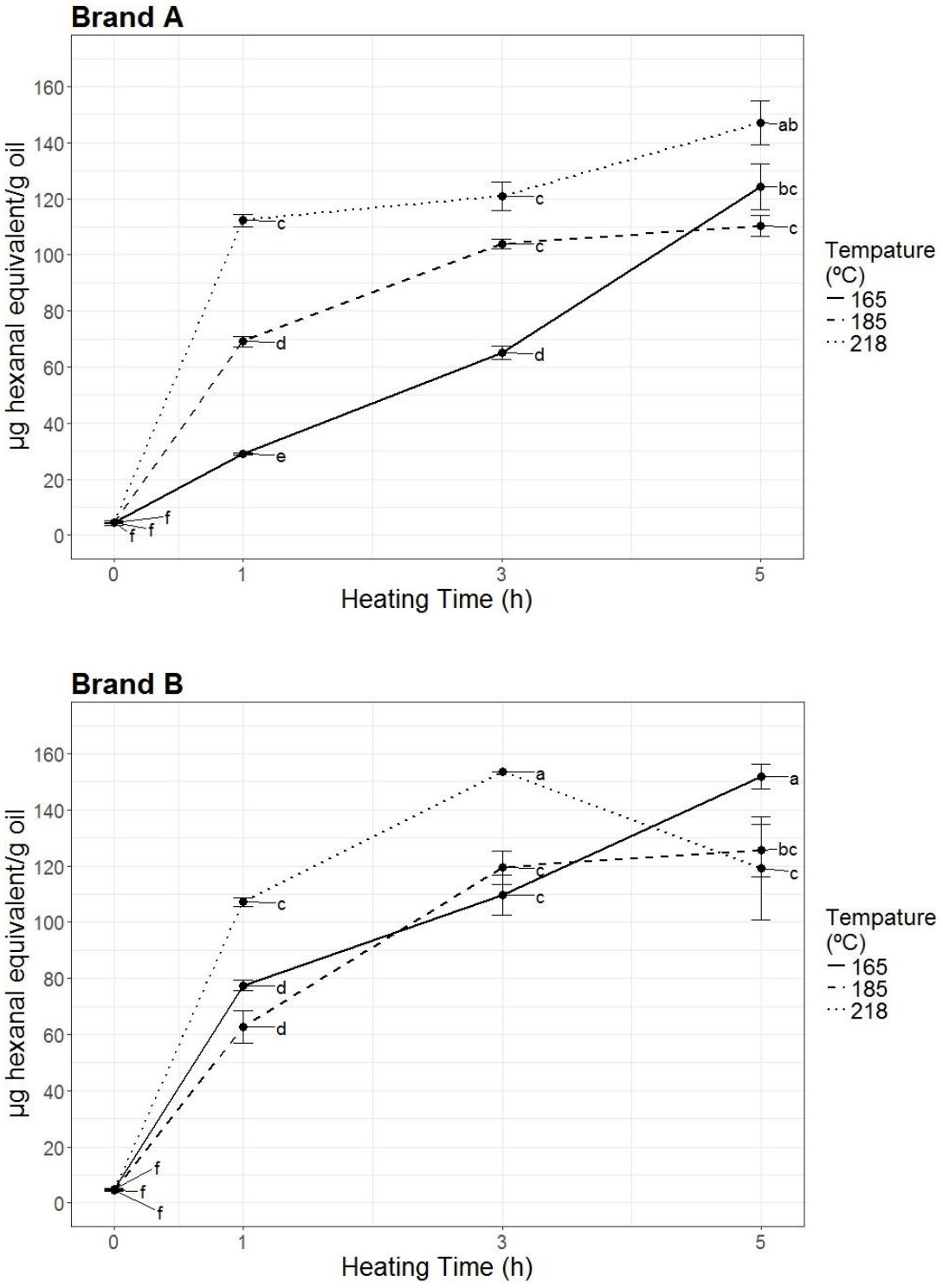

Figure 35: Formation of the sum total of polar lipophilic aldehydes in commercial brand A&B grape seed oil heat treated for up to 5 hours at 165, 185 and 218°C

Figure 32-Figure 35 presented the formation of sum total of polar lipophilic aldehydes and related carbonyl compounds measured as DNPH derivatives and

expressed as hexanal equivalents in coconut, palm, safflower and grape seed oil, respectively. Based on the ANOVA analysis results, only heating time showed significant influence on sum total of polar lipophilic aldehydes. And it can be seen that oils heating at 165 and 185°C, the formation of sum of total polar lipophilic aldehydes grew up with longer heating time; while heating at 218°C, the total polar aldehydes started to decrease after reaching the maximum value at 3 hours. Moreover, only the formation of sum of total polar lipophilic aldehydes of palm oil declined after 1h compared to other oils, and the sum of total polar lipophilic aldehydes of other three oils started to decrease after 3h when heating at 218°C. Different brands of oil showed slightly different pattern, but they presented similar trends.

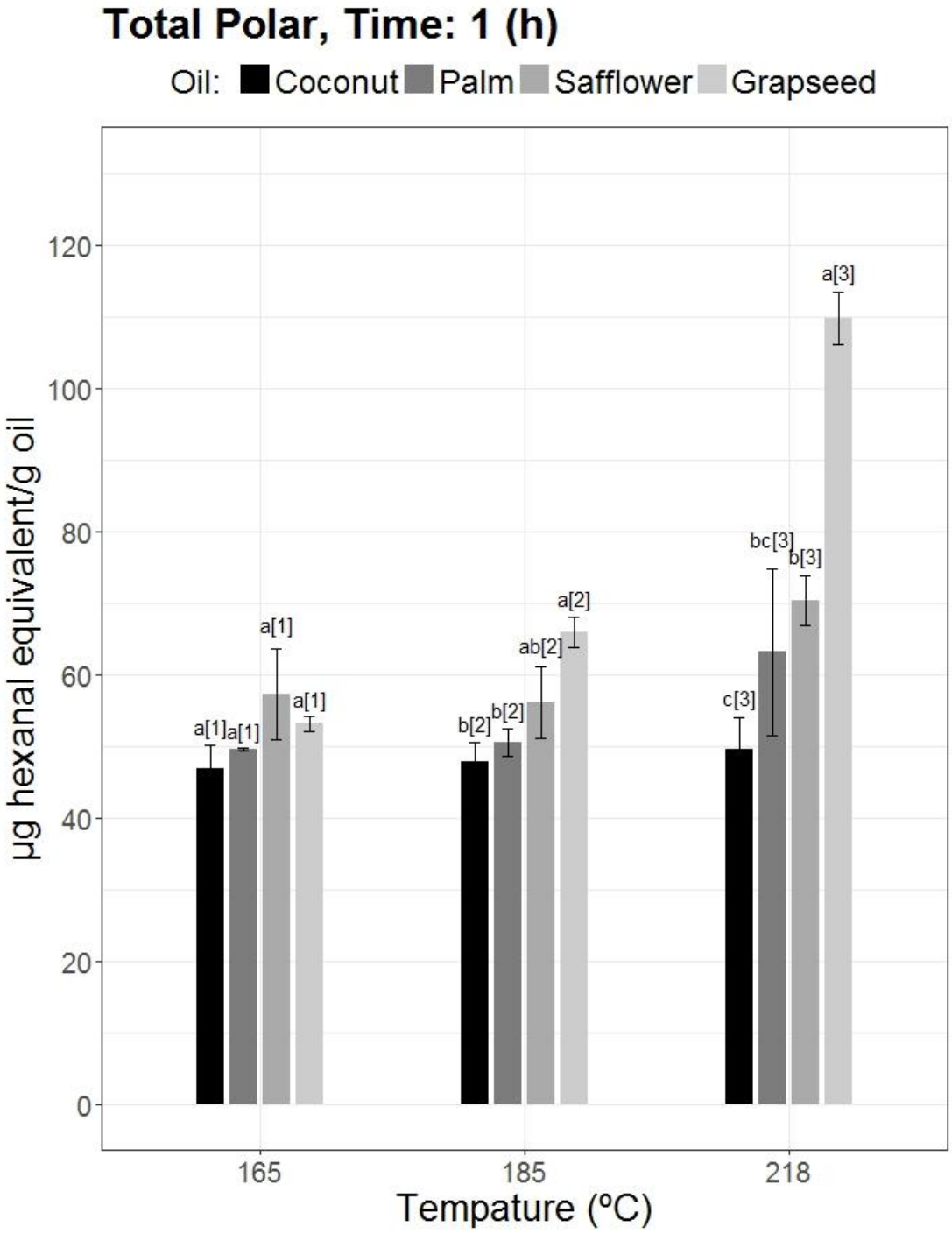

Figure 36: Comparison on sum total of polar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 1hour

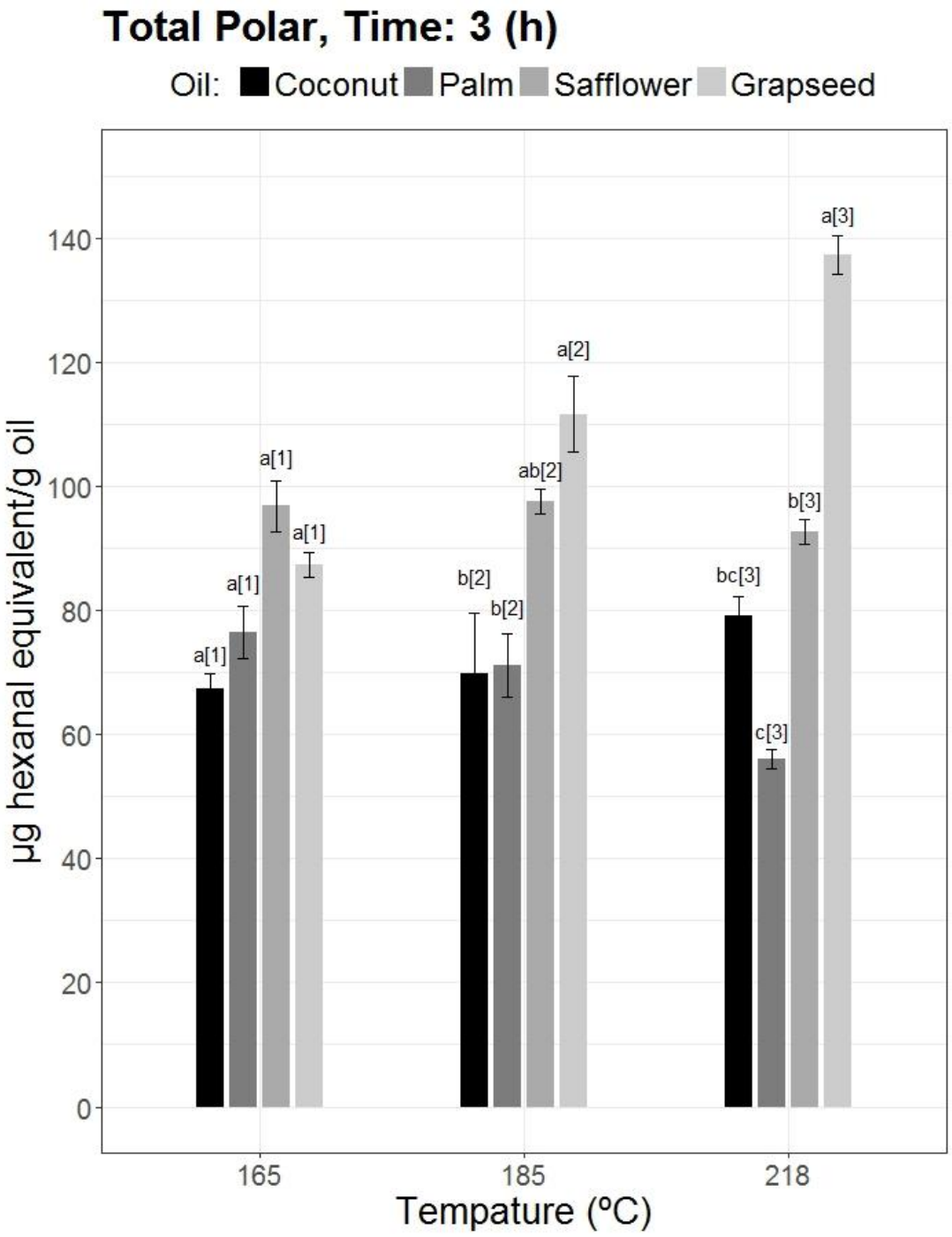

Figure 37: Comparison on sum total of polar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 3 hours

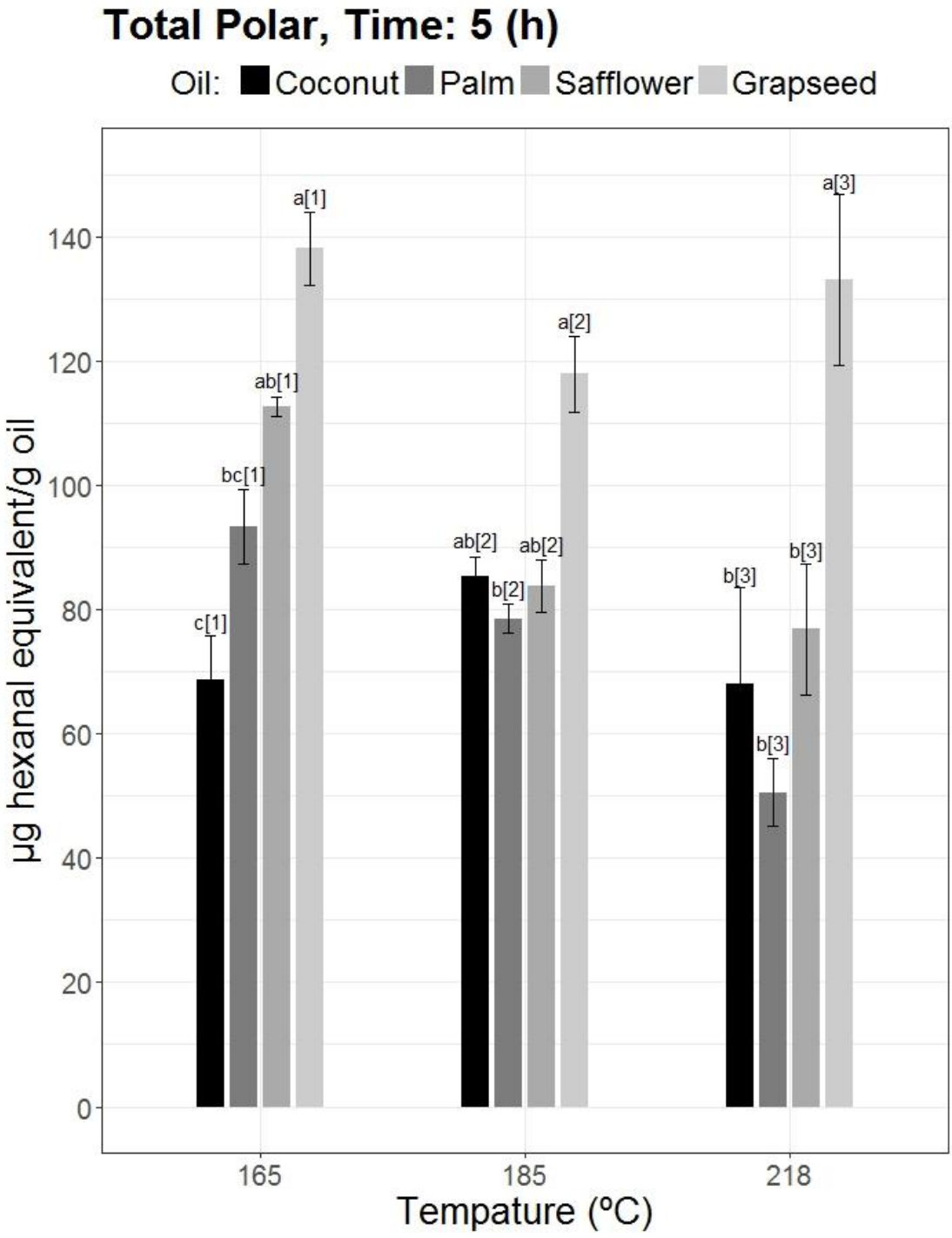

Figure 38: Comparison on sum total of polar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 5 hours

Figure 36-Figure 38 compared the sum total of polar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 1, 3, 5h. We can see that grape seed oil had the highest contents of sum total of polar lipophilic aldehydes than the other three oils at all three heating temperatures, and there was no significant difference among coconut, palm and safflower oil. The result was as expected, because grape seed oil contained the highest percentage of polyunsaturated fatty acids, making it easier oxidize and produce more secondary lipophilic aldehydes. In addition, the formation of sum total of polar lipophilic aldehydes was found to increase with higher heating temperature during 1 hour heating. And heating at 218°C, the formation of sum total of polar lipophilic aldehydes decreased after reaching maximal values due to decomposition of polar aldehydes.

4.7 THE FORMATION OF THE SUM TOTAL OF INDIVIDUAL NONPOLAR LIPOPHILIC ALDEHYDES IN COMMERCIAL COCONUT, PALM, SAFFLOWER AND GRAPE SEED OIL HEAT TREATED FOR UP TO 5 HOURS AT 165, 185 AND 218°C

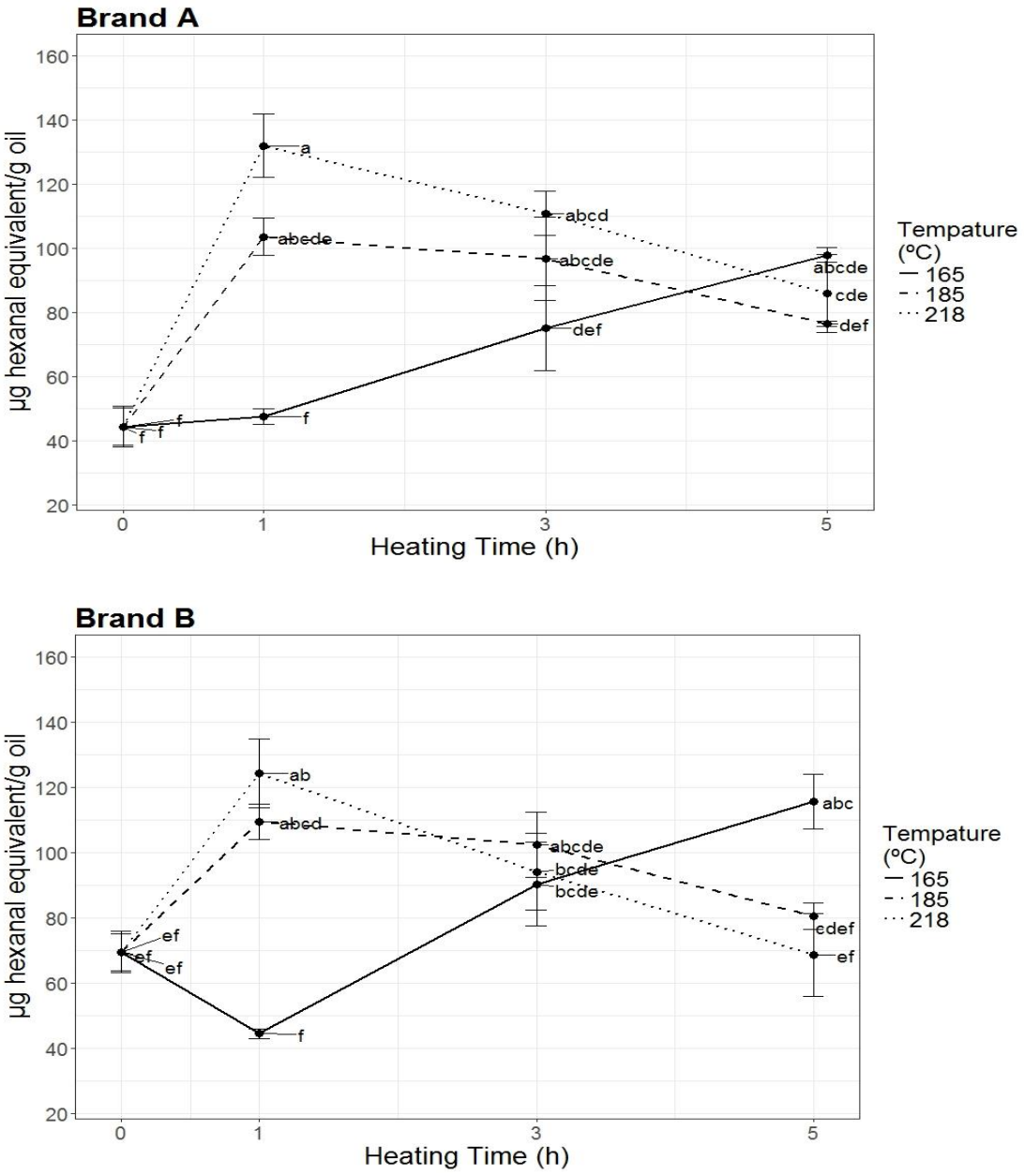

Figure 39: Formation of the sum total of nonpolar lipophilic aldehydes in commercial brand A&B coconut oil heat treated for up to 5 hours at 165, 185 and 218°C

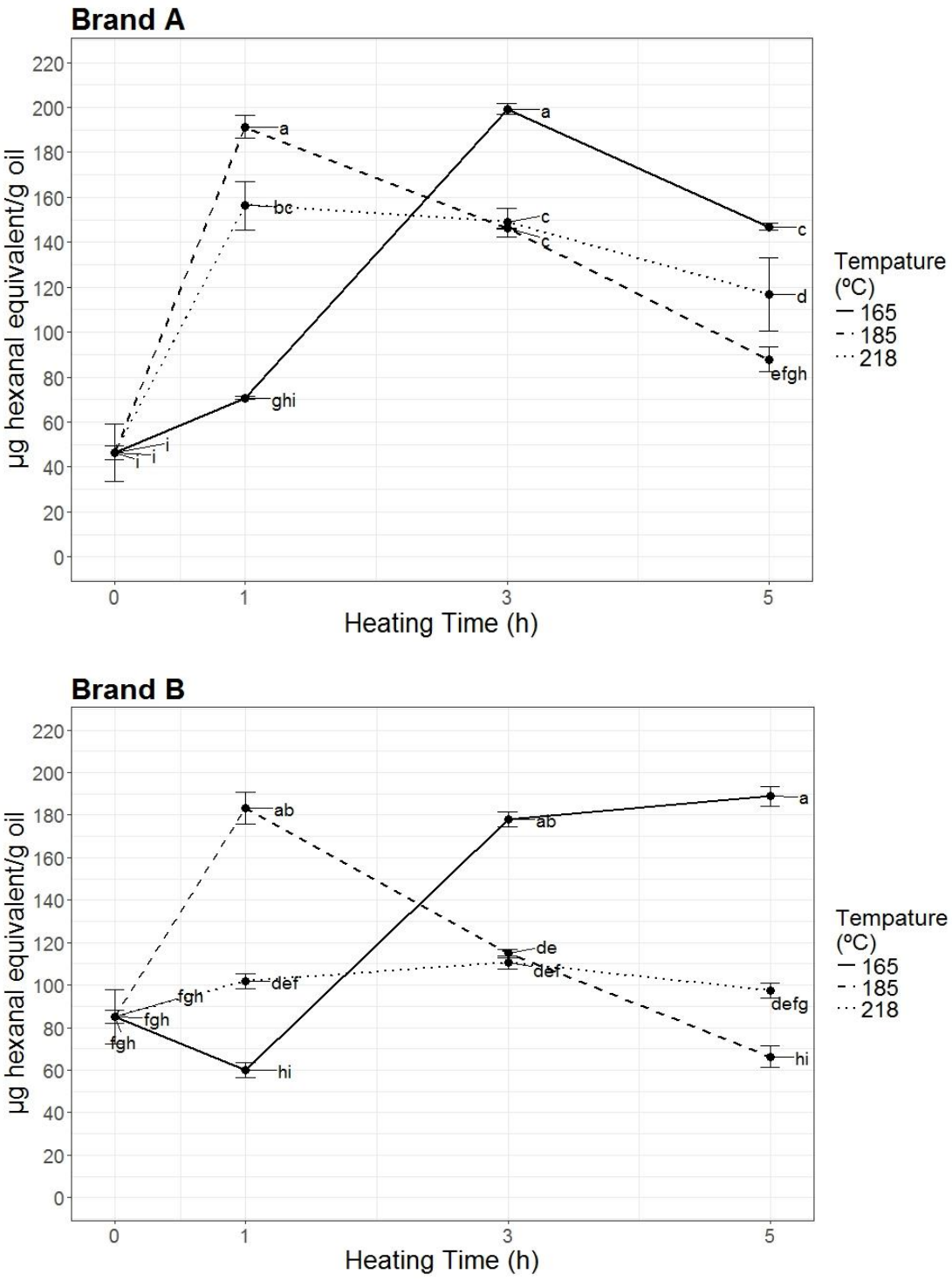

Figure 40: Formation of the sum total of nonpolar lipophilic aldehydes in commercial brand A&B palm oil heat treated for up to 5 hours at 165, 185 and 218°C

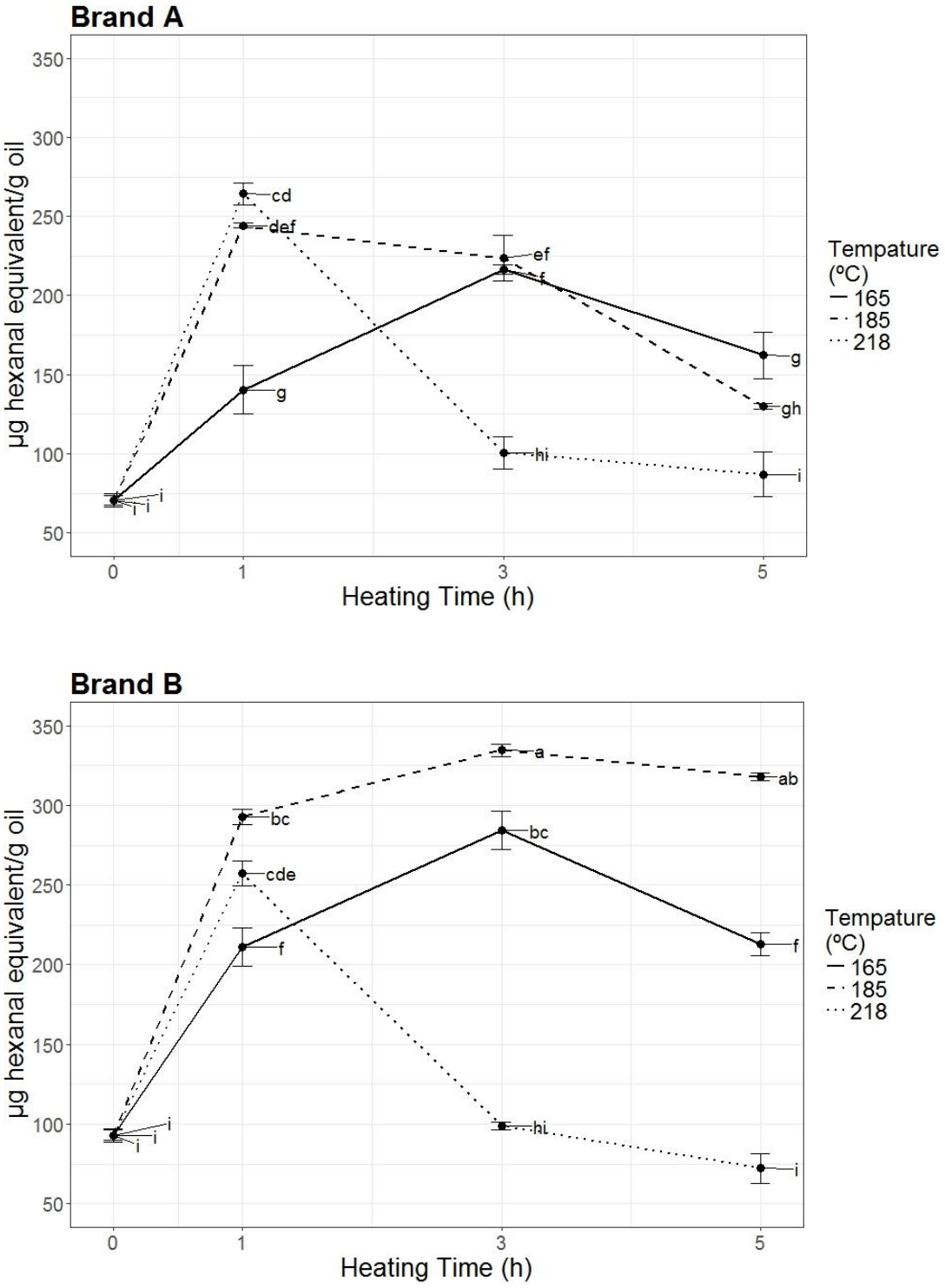

Figure 41: Formation of the sum total of nonpolar lipophilic aldehydes in commercial brand A&B safflower oil heat treated for up to 5 hours at 165, 185 and 218°C

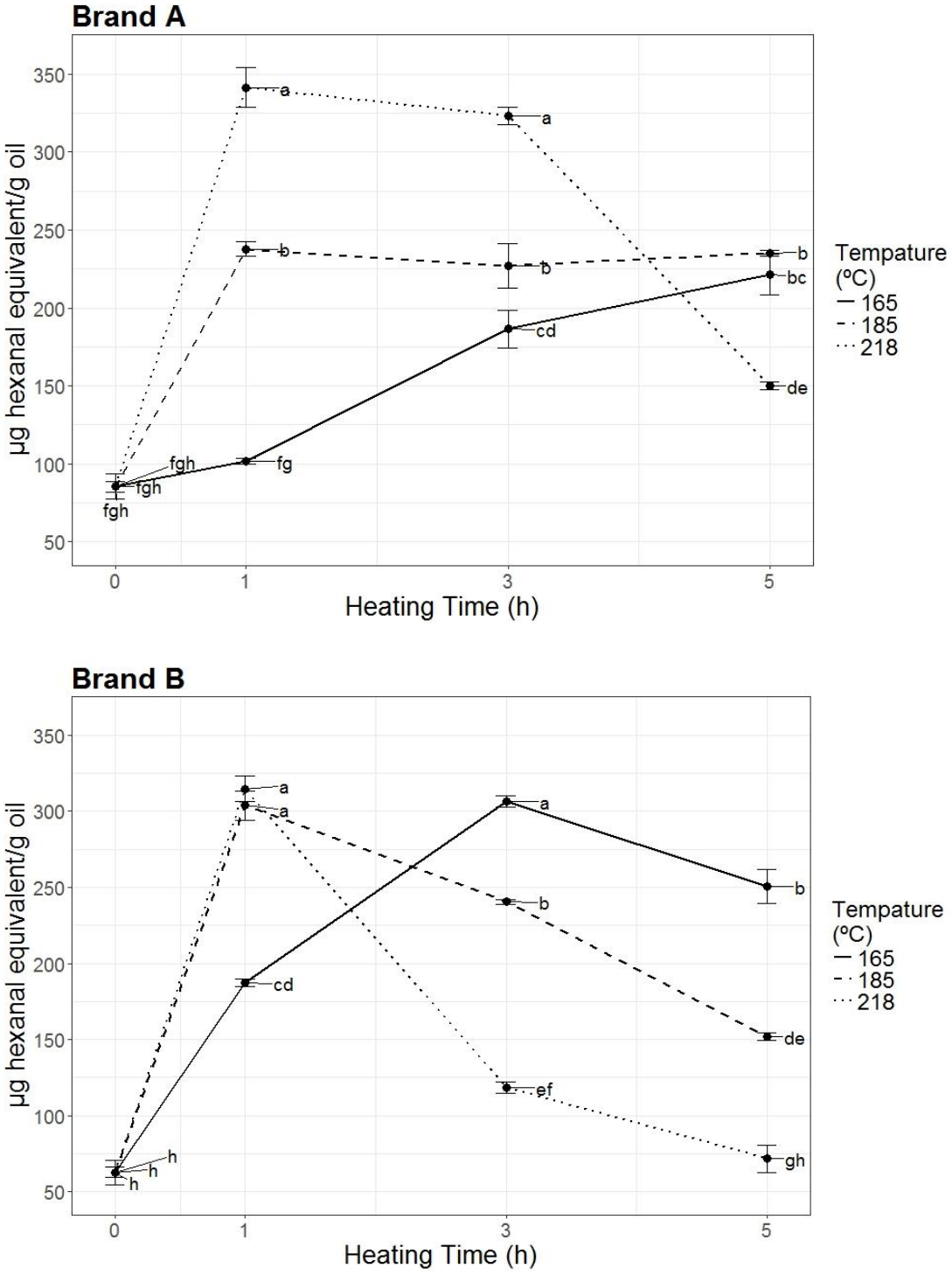

Figure 42: Formation of the sum total of nonpolar lipophilic aldehydes in commercial brand A&B grape seed oil heat treated for up to 5 hours at 165, 185 and 218°C

Figure 39-Figure 42 showed the formation of the sum total of nonpolar lipophilic aldehydes in coconut, palm, safflower and grape seed oil, respectively. According to ANOVA analysis, heating time was a significant factor on influencing the formation of the sum total of nonpolar lipophilic aldehydes. We can find that in our investigated oils, heating at 185 and 218°C, the concentration of the sum total of nonpolar lipophilic aldehydes started to decrease after 1-hour treatment. When heating at 165°C, the sum total of nonpolar lipophilic aldehydes began to decrease after 3-hour treatment. But in particular palm and coconut oil, the trend of formation of total nonpolar aldehydes was totally different. In brand B coconut oil and palm oil, it began to decrease after heating, and when heating for 1 hour, the contents of nonpolar aldehydes grew up gradually with prolonged heating treatment. The difference was due to volatile properties of nonpolar aldehydes, and they were easily evaporated during heat treatment. Results were represented as μg hexanal equivalent/g oil since the individual aldehyde molecular weights are unknown.

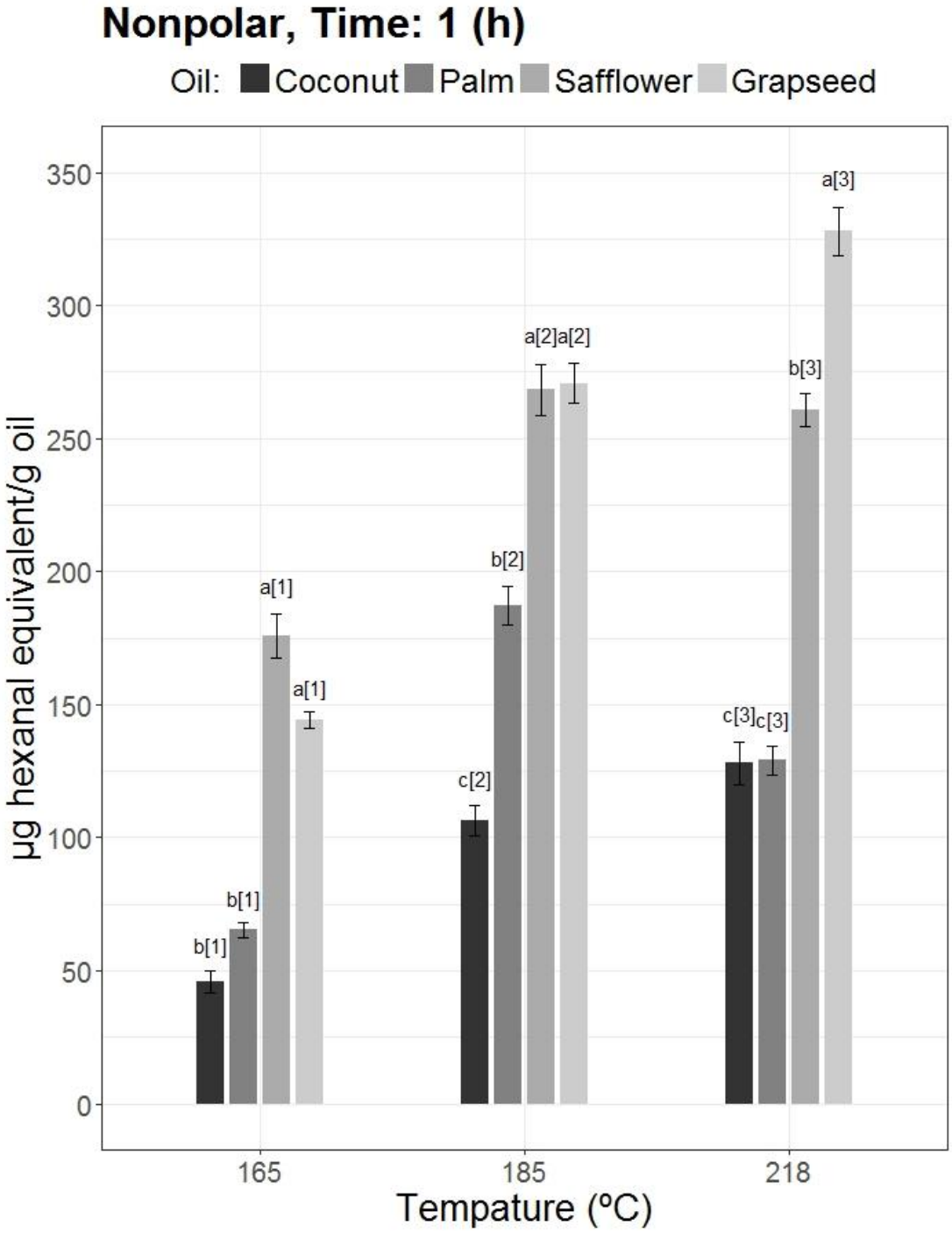

Figure 43: Comparison on sum total of nonpolar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 1hour

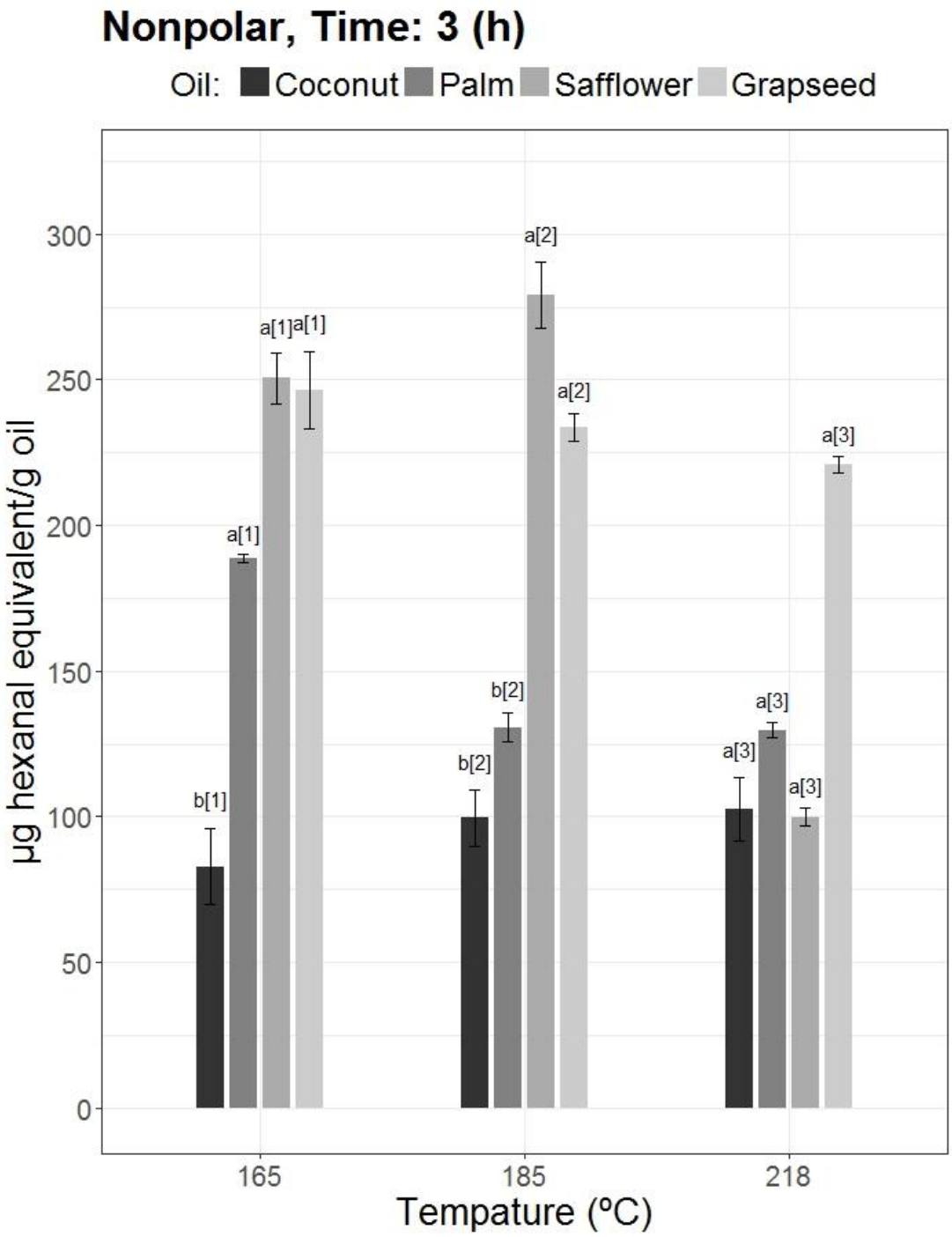

Figure 44: Comparison on sum total of nonpolar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 3hours

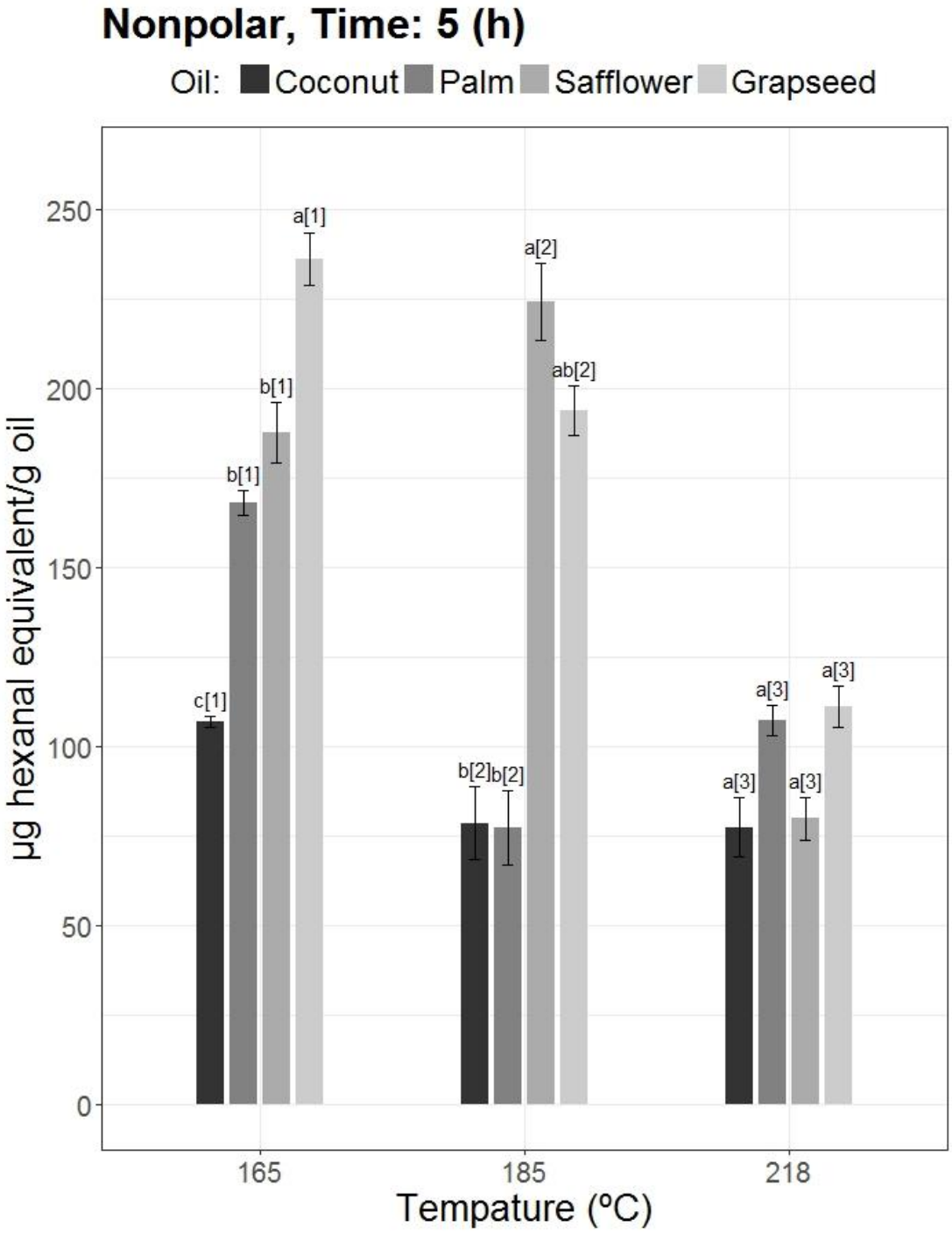

Figure 45: Comparison on sum total of nonpolar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 5 hours

Nonpolar compounds are more volatile than polar compounds. Figure 43-Figure 45 compared the contents of sum total of nonpolar lipophilic aldehydes in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 1, 3, 5h. We can find that for 1 hour heat treatment, the sum total of nonpolar aldehydes increased with higher temperature in all investigated oils. There was no similar trend shown during 3-hour heating. And for heating 5 hours under three temperatures treatment, it's interesting to see that there was an opposite pattern compared to 1-hour heating. Furthermore, under 5-hour and 218°C heat treatment, the formation of sum total of nonpolar lipophilic aldehydes was the lowest compared with other heat treatments due to mostly evaporation of the nonpolar aldehydes.

5 DISCUSSION

As described in the literature (36), the peroxide value between 1 and 5 meq/kg in a product is classified at low oxidation state; peroxide value between 5 and 10 meq/kg is at moderate oxidation and above 10 meq/kg is presented as high oxidation state. The peroxide value of unheated safflower oil obtained in this study is between 5.79 to 8.90 meq/kg, and the peroxide value of unheated grape seed oil is between 4.69 to 5.31 meq/kg. Moreover, the PV of palm oil in the present study was 2.07-2.5 meq/kg. The peroxide value we measured for commercial coconut oil is 1.74-2.69 meq/kg. The PV indicates the concentration of primary oxidation hydroperoxides, which can be influenced by many factors, such as fatty acid distribution of oils, plant cultivars, processing steps and other variables. And the peroxide values measured for all

commercial oils in this experiment were below 10 meq/kg, showing these unheated vegetable oils had low oxidation rate.

The fatty acid distribution of the four vegetable oils investigated in this study is in good accordance with literatures discussed before. However, the oleic acid content of safflower oil is much higher than originally expected, and it may be that there is growing trend in the market to have high monounsaturated fatty acid in concentration for good balance. In addition, by analyzing fatty acid distribution and peroxide value of these four oils, we can see the peroxide value of coconut oil and palm oil are much lower than safflower oil and grape seed oil, which demonstrated that more saturated oils have higher oxidative stability than unsaturated oils.

TBARS assay gave a general information on the oxidation rate and the formation of secondary oxidation products such as aldehydes, ketones and related carbonyl compounds. Results in this study demonstrated that prolonged heating time and higher temperature will induce higher oxidation and higher secondary oxidation products formation. After reaching maximum value, TBARS values started decreasing gradually meaning decomposition and maybe some evaporation of the oxidized products. In general, at 218°C, the formation of secondary oxidation products grew up the fastest compared to the other two lower heating temperatures, especially at the beginning of heating, followed by 185°C heating. And 165°C heating was usually showed lower secondary oxidation products formation, which mostly came from linoleic acid. Oils containing higher unsaturation produces smaller

molecular weight secondary oxidation products and if they are non-polar compounds, they evaporate faster. Moreover, the different oxidation rate among the four oils indicated the effect of saturation degrees of oils. More saturated fatty acids containing oils showed lower secondary oxidation products formation. Temperature was also observed from TBARS values as an important factor to influence lipid oxidation.

4-hydroxy-2-trans-nonenal (HNE), is known as the most toxic compound of the four α, β- unsaturated 4-hydroxyaldehydes, 4-hydroxy-2-trans-hexenal (HHE), 4-hydroxy-2-trans-octenal (HOE), 4-hydroxy-2-trans-nonenal (HNE) and 4-hydroxy-2-trans-decenal (HDE). HNE is highly reactive by Michael addition, which C=C double bonds can react with amino compounds undergoing Michael additions to HNE, and thiol group is added to double bonds by Michael addition. Schiff base occurs with primary amines. All the reactions are relating to several diseases, such as atherosclerosis, stroke, Parkinson's and Alzheimer's diseases (70). In our study, HNE concentration was analyzed in coconut, palm, safflower and grape seed oil heating at 165, 185 and 218°C for 0, 1, 3 and 5 hours in order to investigate influence of temperature, heating time and the compositions of vegetable oils on HNE formation.

Data found in literatures regarding HNE concentration in vegetable oils are scarce, especially for heated oils, and most of data was shown in charts, making it hard to compare with accurate results. According to previous researches, Papastergiadis and his colleagues (71) investigated HNE concentration on unheated oils and food matrix.

The results of HNE concentration in extra virgin olive oil was between 57-117 ng/g sample, and in canola oil was 111 ng/g sample. In addition, Surh and Kwon (72) also demonstrated the average concentration of HNE in sesame oil was 277 μg/kg oil, 80 μg/kg in perilla oil, 130 μg/kg in corn oil, and 499 μg/kg in soybean oil. In our study, the range of HNE concentration in the investigated unheated four vegetable oils was between 0-1.3 μg/g oil. The HNE concentration obtained in our study was in the same range with others in literatures. However, the HNE content investigated in heated oils was very limited in literatures besides published results from this laboratory.

For coconut oil, based on Figure 22, we can see that HNE concentration in both brands was higher when heated at 185°C than heated at 165°C. And for 218°C, more decomposition happened, making it hard to find a typical trend. There was no significant difference between two brands of coconut oil under same heat treatment. In terms of 1 and 3-hour heating, there was no significant difference among three temperatures, especially shown in brand B coconut oil.

For palm oil, based on Figure 24, there was significant difference found in two brands of palm oils under same heat treatment. We found that HNE concentration was higher when heating at 185°C compared to heating at 165°C. When heating at 218°C, HNE formation declined due to some decompositions. Brand B palm oil showed a growing trend with higher temperature, even at 218°C under 3 hours heating, but there was not significant difference on HNE concentration between 185 and 218°C heating.

For safflower oil, based on Figure 26, there was significant difference on HNE concentration between two brands of safflower oil under 1-hour heating treatment. And according to Figure 11, the antioxidant capacity results from four investigated oils, it was seen that there was significant difference between two brands of safflower oils, which may be a factor that caused HNE concentration difference. Moreover, by comparing brand A and brand B safflower oil, brand B safflower oil presented higher HNE concentration when heating at 165°C. In addition, HNE accumulated rapidly in brand B safflower oil, it's because brand B safflower oil contained significantly higher linoleic acid than brand A safflower oil (Figure 6). In general, as the same trend discussed before, higher temperature induced more HNE formation. However, when heating at 218°C, HNE started to decompose under certain period of time.

Contrary to the other oils, grape seed oil, (Figure 28), it can be seen that with higher heating temperature, at 218°C, HNE still increased gradually, which was a big difference compared to the other three oils. This difference could be due to the highly polyunsaturated fatty acids contained in grape seed oil, which makes it more easily oxidized and able to produce more HNE formation for a longer period of time even when some decomposition was taking place at the same time.

A previous published paper from this laboratory also investigated the temperature dependence of HNE formation in corn, soybean and butter oil (24). It compared HNE concentration under 190 and 218°C heat treatment, and results showed higher HNE formation at 218°C than heating at 190°C for 30 min in all three oils. In the present

study, 165, 185 and 218°C heating temperatures were investigated on HNE formation and heating periods were 1, 3 and 5 hours. We found that HNE formation increased with higher heating temperature, such as from 165°C to 185°C. However, when heating at 218°C, most oils showed declining trend on HNE concentration under 3 and 5-hours heat treatment. For 1-hour at 218°C, it showed a similar pattern than oils previously reported (24). Therefore, the heating time and the temperature were both important factors that had influence on HNE formation in general.

Based on the statistics conducted using ANOVA results from present experiments, it showed that the combination of the three factors, such as unsaturation, temperature, and heating time, resulted in significantly influence on HNE formation. For the four oils, linoleic concentrations of coconut, palm, safflower and grape seed oil were 5.8%, 11.2%, 15.0% and 62.3%, respectively. When heating at 185°C, grape seed oil was shown to have the highest HNE concentration (73.6 µg HNE/g oil for 3h), followed by safflower oil (27.5 µg HNE/g oil for 3h), palm oil (19.5 µg HNE/g oil for 3h) and coconut oil (7.5 µg HNE/g oil for 3h). The sequence of HNE formations were similar as the order of linoleic contents of four oils. This was as expected, because linoleic acid is known to be a precursor of HNE. In the present study, the various brands of commercial oils showed some different pattern on HNE formation. This may be due to antioxidant capacity difference, fatty acid composition variance, and possibly other factors including oil processing. Even though the data showed no significant differences between most of oils, our quantities of sample was very small, it's hard to

use Tukey test to identify the discrepancies. Furthermore, in general, it was found that HNE formation decreased faster under very higher temperature (218°C) and longer heat treatment (5h), this may be the result of the faster decomposition than the formation of HNE at this temperature. In general, we can see that in all investigated oils, HNE concentration was higher at temperature 185°C compared to at 165°C. Heating at 218°C, more and faster HNE production and also degradation occurred. Mass spectrometry was conducted and HNE-DNPH was confirmed in all investigated oils.

Last but not least, sum total of individual polar aldehydes was measured and found sensitive to heating time and temperature. In general, with longer heating time and higher temperature, the formation of sum total of individual polar aldehydes increased. But when the heating temperature was too high and heating time was long enough, total polar aldehydes started to degrade, resulting in less contents of sum total of polar aldehydes detected. Due to volatile properties of some nonpolar aldehydes under heat treatment, the formation of sum of total of individual nonpolar aldehydes began decreasing when reaching certain increased heating time and temperature.

6 CONCLUSION

In this study, the HNE formation was measured in the four newly developed commercial vegetable oils, such as coconut oil, palm oil, safflower oil and grape seed oil. It was found that HNE formation was mostly temperature and heating time

dependent. Based on the present results of this study, lower temperature and shorter heating time should be used to minimize the formation of HNE and toxic lipid peroxidation products. Vegetable oils which have lower level of linoleic acid concentration produce less toxic HNE and the total polar aldehydes, such as coconut oil in the present study, which suggest that as a good oil source for cooking.

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8 APPENDIX:

8.1 ANOVA

Analysis of variances (ANOVA) is a collection of statistical methods to analyze the differences among group means. As its name suggests, ANOVA helps to achieve this by assessing different variance components, namely between-group variability and within-group variability. Between-group variability can be obtained from sum of squares of the differences between group mean and grand mean for each sample. Within-group variability can be obtained from sum of squares of the differences between response and grand mean for each sample. Intuitively, if between-group variability is greatly larger than within-group variability, we can conclude that the group differences are significantly large. Fortunately, a well-known statistical finding is that between-group variability/within-group variability follows a specific F distribution, so that we can easily construct an F test. Then we can compute the p-value, defined as the probability that a value larger than between-group variability/within-group variability appears under the F-distribution. In compliance with our intuition, a big value of between-group variability/within-group variability would results in a small p-value and thus imply statistical significant differences among groups.

In my experiments, between-group variability come from three sources, 'Brand', 'Temperature' and 'Time'. We can separate our samples into groups with tags 'Brand A', 'Brand B', or into groups with tags '165℃', '185℃', '218℃', or into groups with tags '1h', '3h' and '5h'. For each of the three sources, we can compute a between-group variability, by computing the associated sum of squares divided by its degrees of freedom (number of groups – 1). The within-group variability part is always the residual sum of squares divided by its degree of freedom (number of samples – number of groups). Now we can compute different F values for each source and thus under different F-tests, three p-values are computed associated with each character.

ANOVA analysis were done for HNE concentration, Total Polar concentration and Non-Polar concentration and the results are as follows.

1. HNE:

1) Safflower

Analysis of Variance Table

Response: Concentration

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Brand	1	1108.8	1108.8	7.6920	0.00831 **
Temperature	2	1203.6	601.8	4.1748	0.02237 *
Time	3	12037.8	4012.6	27.8364	5.556e-10 ***
Residuals	41	5910.1	144.1		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

2) Grapeseed

Analysis of Variance Table

Response: Concentration

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Brand	1	969	969.3	4.0837	0.04986 *
Temperature	2	6149	3074.3	12.9521	4.368e-05 ***
Time	3	80643	26880.9	113.2502	< 2.2e-16 ***
Residuals	41	9732	237.4		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

3) Coconut

Analysis of Variance Table

Response: Concentration

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Brand	1	0.08	0.075	0.0388	0.8449
Temperature	2	1.88	0.938	0.4838	0.6199
Time	3	491.22	163.740	84.4382	<2e-16 ***

```
Residuals 41  79.51   1.939
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

4) Palm Oil

```
Analysis of Variance Table

Response: Concentration
---
            Df  Sum Sq Mean Sq F value    Pr(>F)
Brand        1    6.8    6.83   1.2026    0.2792
Temperature  2  178.3   89.16  15.7105 8.607e-06 ***
Time         3 3951.7 1317.23 232.0964 < 2.2e-16 ***
Residuals 41  232.7    5.68
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2. Total Polar

1) Safflower

```
Analysis of Variance Table

Response: Concentration
---
            Df  Sum Sq Mean Sq F value    Pr(>F)
Brand        1    594   593.6   3.6914 0.06166 .
Temperature  2    527   263.4   1.6382 0.20679
Time         3  59958 19986.2 124.2845 < 2e-16 ***
Residuals 41   6593   160.8
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2) Grapseed

```
Analysis of Variance Table

Response: Concentration
---
            Df  Sum Sq Mean Sq F value    Pr(>F)
```

```
Brand       1   1763    1763   5.3607  0.025677 *
Temperature  2   5876    2938   8.9339  0.000602 ***
Time        3 110452   36817 111.9496 < 2.2e-16 ***
Residuals 41  13484     329
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

3) Palm

```
Analysis of Variance Table
Response: Concentration
            Df  Sum Sq Mean Sq F value    Pr(>F)
Brand       1   1044  1044.4  6.6757 0.01344 *
Temperature  2   1255   627.4  4.0103 0.02566 *
Time        3  38590 12863.2 82.2206 < 2e-16 ***
Residuals 41   6414   156.4
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

4) Coconut

```
Analysis of Variance Table
Response: Concentration
            Df  Sum Sq Mean Sq F value    Pr(>F)
Brand       1    216   215.9  1.1213    0.2958
Temperature  2    213   106.3  0.5522    0.5799
Time        3  38063 12687.6 65.8926 9.868e-16 ***
Residuals 41   7895   192.6
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

3. Non-Polar:

1) Safflower:

Analysis of Variance Table

Response: Concentration

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Brand	1	28082	28082	9.3267	0.0039570	**
Temperature	2	54913	27456	9.1190	0.0005294	***
Time	3	163364	54455	18.0859	1.247e-07	***
Residuals	41	123446	3011			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

2) Grapeseed:

Analysis of Variance Table

Response: Concentration

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Brand	1	1808	1808	0.3978	0.5317	
Temperature	2	2534	1267	0.2788	0.7581	
Time	3	223230	74410	16.3711	3.815e-07	***
Residuals	41	186353	4545			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

3) Palm

Analysis of Variance Table

Response: Concentration

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Brand	1	183	182.5	0.1092	0.7427907	
Temperature	2	1576	788.0	0.4712	0.6275722	
Time	3	45410	15136.6	9.0523	0.0001014	***

```
Residuals 41  68557  1672.1
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

4) Coconut

```
Analysis of Variance Table

Response: Concentration
---
            Df  Sum Sq Mean Sq F value    Pr(>F)
Brand        1   515.5   515.5  1.0877 0.3030874
Temperature  2  2733.4  1366.7  2.8839 0.0673232 .
Time         3 11439.0  3813.0  8.0458 0.0002485 ***
Residuals 41 19430.4   473.9
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

8.2 TABLES OF TOTAL POLAR ALDEHYDES AND NONPOLAR ALDEHYDES AND THE SUM OF TOTAL POLAR ALDEHYDES AND NONPOLAR ALDEHYDES

Table 12: Total polar (TP) results and total nonpolar (TNP) results and the sum of total polar and nonpolar results of four oils heated at 165°C for 0,1,3,5h (1µg Hexanal equivalent/g oil)

	Time (h)	Brand A coconut oil	Brand B coconut oil	Brand A palm oil	Brand B palm oil	Brand A safflower oil	Brand B safflower oil	Brand A grape seed oil	Brand B grape seed oil
TP	0	8.34	0	0.05	4.2	4.05	9.85	4.43	4.97
	1	45.38	48.16	46.87	52.3	49.98	64.51	29	77.38
	3	54.11	80.8	87.35	65.64	85.58	108.05	64.95	109.76
	5	49.02	88.45	63.96	93.78	102.65	122.52	124.35	151.91
TNP	0	44.4	69.4	46.3	85.1	70.6	93	85.5	62.7
	1	47.6	44.4	70.7	60.1	132.8	211.1	101.7	187.1
	3	75.2	90.3	199.2	178	216.6	278.5	189	306.4
	5	87.8	115.5	171.2	188.8	167.2	212.9	224.1	250.6
TP+ TNP	0	52.74	69.4	46.35	89.3	74.65	102.85	89.93	67.67
	1	92.98	92.56	117.57	112.4	182.78	275.61	130.7	264.48
	3	129.31	171.1	286.55	243.64	302.18	386.55	253.95	416.16
	5	136.82	203.95	235.16	282.58	269.85	335.42	348.45	402.51

Table 13: Total polar (TP) results and total nonpolar (TNP) results and the sum of total polar and nonpolar results of four oils heated at 185°C for 0,1,3,5h

	Time (h)	Brand A coconut oil	Brand B coconut oil	Brand A palm oil	Brand B palm oil	Brand A safflower oil	Brand B safflower oil	Brand A grape seed oil	Brand B grape seed oil
TP	0	8.34	0	0.05	4.2	4.05	9.85	4.43	4.97
	1	44.98	50.68	57.45	43.56	49.68	62.49	69.05	62.8
	3	84.57	55.12	87.88	54.48	91.75	103.31	103.88	119.34
	5	110.06	60.68	90.4	66.66	85.62	81.96	110.27	125.51
TNP	0	44.4	69.4	46.3	85.1	70.6	93	85.5	62.7
	1	103.1	109.5	194.8	183.3	244.4	292.6	240.3	303.6
	3	96.7	108.2	146.2	114.9	223.7	327.7	228.6	240.5
	5	82.4	82.1	96.4	66.3	129.9	318	235.1	152.1
TP + TNP	0	52.74	69.4	46.35	89.3	74.65	102.85	89.93	67.67
	1	148.08	160.18	252.25	226.86	294.08	355.09	309.35	366.4
	3	181.27	163.32	234.08	169.38	315.45	431.01	332.48	359.84
	5	192.46	142.78	186.8	132.96	215.52	399.96	345.37	277.61

Table 14: Total polar (TP) results and total nonpolar (TNP) results and the sum of total polar and nonpolar results of four oils heated at 218°C for 0,1,3,5h

	Time (h)	Brand A coconut oil	Brand B coconut oil	Brand A palm oil	Brand B palm oil	Brand A safflower oil	Brand B safflower oil	Brand A grape seed oil	Brand B grape seed oil
TP	0	8.34	0	0.05	4.2	4.05	9.85	4.43	4.97
	1	48.24	50.79	68.97	57.32	64.68	75.95	112.23	107.13
	3	89.22	68.84	65.89	46.13	83.51	101.79	120.9	153.54
	5	70.03	66.14	53.81	47.37	96.95	56.71	146.96	119.11
TNP	0	44.4	69.4	46.3	85.1	70.6	93	85.5	62.7
	1	139.5	124.3	159.7	101.9	264.3	257.6	341.3	314.6
	3	110.8	94.1	149.2	110.5	100.5	98.9	323.3	118.4
	5	85.9	71.9	116.8	97.3	87.1	72.2	150.1	71.8
TP + TNP	0	52.74	69.4	46.35	89.3	74.65	102.85	89.93	67.67
	1	148.08	160.18	252.25	226.86	294.08	355.09	309.35	366.4
	3	181.27	163.32	234.08	169.38	315.45	431.01	332.48	359.84
	5	192.46	142.78	186.8	132.96	215.52	399.96	345.37	277.61